UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

AGING PATHOLOGY IN SPRAGUE DAWLEY RATS: BACKGROUND LESIONS AND COMPARATIVE STUDY BETWEEN WILD TYPE AND TRANSGENIC RATS WITH NEURONAL OVEREXPRESSION OF HUMAN ADENOSINE A_{2A} RECEPTORS

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Não tenhamos pressa, mas não percamos tempo

José Saramago

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Abstract

Aging is a complex phenomenon defined as a time-dependent functional decline, progressive loss of physiological integrity and progressive increase in disease susceptibly. Adenosine A_{2A} receptors (A2AR) are G protein-couple receptors that, upon binding of adenosine, lead to different transducing signals. Although having a protective effect, A2AR also play an important role in neurodegenerative disorders and are upregulated in the brain of Alzheimer and Parkinson patients. Previous studies from our collaborators showed that transgenic rats with neuronal overexpression of human A2AR (Tg (CaMKIIhA2AR)) have depressive-like behavior, impaired hypothalamic-pituitary-adrenal (HPA) axis and, as a result of this, increased levels of circulating corticosteroids. The aim of this work was to evaluate, by histopathology, the impact of the neuronal overexpression of human A2AR in the onset of specific or age-associated lesions in transgenic Sprague Dawley rats. Comprehensive necropsy and histopathology were performed in 37 Wild-type (Wt) and 39 transgenic (Tg) rats, at specific time-points, ranging from 12 to 126 weeks of age. Univariate and multivariate statistical analysis were performed to investigate the association between the phenotype and genotype. Briefly we found that Tg rats are 2.7 times more likely to develop systemic pathology than Wt rats [Odds ratio (OR) 2.745, IC 95% 1.0.07-6.997; ($p<0.05$)]. In the heart cardiomyopathy was the most frequent lesion both in Wt and Tg rats, and its incidence did not differ between groups [OR 0.82; IC 95% 0.315-2.139, ($p>0.05$)]. In blood vessels, mineralization was the most frequent lesion and Tg rats were 5.5 times more likely to develop this lesion than Wt [OR 5.486, IC 95% 1.776- 17.074; ($p<0.05$)]. In lung, alveolar histiocytosis and alveolar septa mineralization were the most frequent lesions and Tg rats were 7.7 times more likely to develop lung pathology than Wt [OR 7.7, IC 95% 1.604-37.19; ($p<0.05$)]. In kidney, chronic progressive nephropathy was the most frequent lesion both in Wt and Tg rats, and its incidence did not differ between groups [OR 2.5, IC 95% 0.919-6.923; ($p>0.05$)]. Regarding adrenal gland pathology, vacuolation of the cortical cells was the most frequent lesion and Tg rats were 4.3 times more likely to develop this pathology than Wt [OR 4.3, IC 95% 1.156-16.248; ($p<0.05$)]. Mammary fibroadenoma was the most common tumor in our sample, being observed in one Wt and five Tg rats. Even in cases where no difference was seen when comparing Tg and Wt rats, all lesions found in our study were age-associated lesions, typical for this species, and their incidence correlated with age. Our results show a clear correlation between increased

A2AR signaling in the brain and accelerated aging, in our sample, and although herein we did not explore the precise mechanism(s) through which this occurs, it could be linked to the fact that Tg rats have HPA-axis dysfunction and increased circulating levels of corticosterone, which translated into chronic stress. To our knowledge, this is the first study to characterize the systemic repercussion of neuronal overexpression of adenosine A_{2A} receptors, which is seen in several degenerative disorders during the aging process.
Key words: Adenosine A_{2A} receptors; aging; animal models; histopathology; phenotyping; Sprague-Dawley rat; transgenic

Resumo

O envelhecimento é um fenómeno complexo definido como um declínio funcional dependente do tempo, com perda progressiva da integridade fisiológica e aumento gradual da suscetibilidade a doenças. Os recetores de adenosina A_{2A} (A2AR) são recetores acoplados à proteína G cuja ligação à adenosina leva a diferentes sinais de transdução. Apesar do seu efeito protetor, os recetores de adenosina A_{2A} têm também um papel crítico em doenças neurodegenerativas e estão sobre expressos no cérebro de doentes de Alzheimer e Parkinson. Estudos recentes demostraram que ratos transgénicos com sobre expressão neuronal de A2AR (Tg (CaMKIIhA2AR)) apresentam comportamento depressivo e disfunção do eixo hipotálamo-hipófise-adrenal, resultando em níveis elevados de corticosteroides circulante. O objetivo deste estudo foi avaliar, por histopatologia, o impacto da sobre expressão neuronal de A2AR no fenótipo de envelhecimento de vários órgãos e sistemas, em ratos Sprague Dawley. Foi efetuada a necrópsia compreensiva e histopatologia em 37 ratos "Wild type" (Wt) e 39 transgénicos (Tg) com idade variável entre as 12 e as 126 semanas. Foi efetuada análise estatística univariável e multivariável para investigar a associação entre o fenótipo e o genótipo. Resumidamente, descobrimos que os ratos Tg foram 2.7 vezes mais suscetíveis a desenvolver patologia sistémica, comparativamente aos Wt [Odds ratio (OR) 2.745, IC 95% 1.0.07-6.997; ($p<0.05$)]. No coração, a lesão mais frequentemente diagnosticada foi cardiomiopatia e a sua incidência não variou entre Wt e Tg [OR 0.82; IC 95% 0.315-2.139, ($p>0.05$)]. Nos vasos sanguíneos, a lesão mais frequente foi a mineralização da parede, sendo que os ratos Tg foram 5.5 vezes mais suscetíveis a desenvolver esta lesão que os Wt [OR 5.486, IC 95% 1.776- 17.074; ($p<0.05$)]. Relativamente ao pulmão, as lesões mais frequentes foram a histiocitose alveolar e a mineralização dos septos alveolares. Os ratos Tg foram 7.7 vezes mais suscetíveis a desenvolver estas lesões que os Wt [OR 7.7, IC 95% 1.604-37.19; ($p<0.05$)]. Relativamente ao rim, a lesão mais frequente foi a nefropatia crónica progressiva e a sua incidência não variou entre os ratos Wt e Tg [OR 2.5, IC 95% 0.919-6.923; ($p>0.05$)]. Relativamente às adrenais, a lesão mais frequente foi a vacuolização das células da cortical e os ratos Tg foram 4.3 vezes mais suscetíveis para o desenvolvimento desta lesão que os Wt [OR 4.3, IC 95% 1.156-16.248; ($p<0.05$)]. Fibroadenoma mamário foi o tumor mais frequente, tendo sido observado em um rato Wt e cinco ratos Tg. Mesmo em casos onde não foi observada diferença entre ratos Wt e Tg, todas as lesões encontradas neste estudo são lesões muitas vezes associadas ao envelhecimento, típicas desta espécie e a sua incidência correlacionou-se com a idade.

Os nossos resultados mostram uma relação clara entre a sobre expressão neuronal de A2AR e envelhecimento acelerado na nossa amostra, e apesar de não termos explorado os mecanismos específicos para tal acontecimento, poderá estar ligado ao facto dos ratos Tg terem disfunção do eixo hipotálamo-hipófise-adrenal e níveis elevados de corticosterona, o que se traduz em stress crónico. A conhecimento dos autores, este é o primeiro estudo a caracterizar as repercussões sistémicas da sobre expressão neuronal de A2AR, que é observada em várias doenças degenerativas durante o envelhecimento.

Palavras-chave: Envelhecimento; fenotipagem; histopatologia; modelos animais; ratos Sprague-Dawley; recetores de adenosina A_{2A}; transgénico.

List of Abbreviations:

A2AR: adenosine A_{2A} receptor

ACTH: adrenocorticotropic hormone

ATP: adenosine triphosphate

AVP: arginine vasopressin

AMP: adenosine monophosphate

BALT: bronchial associated lymphoid tissue

BN: Brown Norway

cAMP: cyclic AMP

CRH: corticotropin release hormone

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated 9

DNA: deoxyribonucleic acid

ESC: embryonic stem cells

F334: Fisher 344

FDA: Food and Drug Administration

GEM: genetically engineered models

GEMM: genetically engineered mouse models

GH: growth hormone

GOI: gene of interest

HPA: hypothalamus-pituitary-adrenal

IC: interval of confidence

IGF-1: insulin growth factor 1

IL: interleukin

iMM-JLA: Instituto de Medicina Molecular- João Lobo Antunes

KO: knock out

mTOR 1: mammalian target of rapamycin 1

pESK: extracellular signal-regulated kinase

PKA: protein kinase A

SD: Sprague Dawley

SASP: senescence associated secretory phenotype

TG: transgenic

TGF-β transforming growth factor-β

TNFα: tumor necrosis factor α

VEGF: vascular endothelial growth factor

Wt: Wild-type

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CHAPTER I- INTERNSHIP REPORT

The final internship for the acquisition of the integrated master's in veterinary medicine (MIMV) degree by the Faculty of Veterinary Medicine- University of Lisbon took place in three different institutions, making a total of 1217 hours. From September to February, the internship took place in the Laboratory of Histology and Comparative Pathology of the Instituto de Medicina Molecular - João Lobo Antunes (iMM-JLA), Faculty of Medicine, University of Lisbon (630 hours) and in the section of Veterinary Anatomic Pathology of the Faculty of Veterinary Medicine, University of Lisbon (157 hours). From March to May the internship took place at the department of Comparative Medicine and Pathology of the Memorial Sloan Kettering Cancer Center in Manhattan, New York City (430 hours).

At IMM, the student was under the guidance of Doctor Tânia Carvalho (DVM, PhD) and had a broad spectrum of functions such as necropsy of animals from the animal facility; gross description and trimming of necropsy tissues; histopathologic analysis of tissues from mice, rats and zebrafish from several research groups, with special focus on aging, cancer biology, parasitology, stem cell biology, among others. Special attention was given to the Adenosine $_{2A}$ receptor overexpression rat model and to cancer biology projects on leukemia and on central nervous system metastasis. The student also gained experience in digital pathology techniques; basic translational science procedures and in organizing research projects in a comparative pathologist point of view.

At the Faculty of Veterinary Medicine, the student was under the orientation and supervision of Professor Conceição Peleteiro (DVM, PhD, Dip. European College of Veterinary Pathology) and performed small and large animal necropsies, gross pathology and trimming of surgical specimens and biopsies and subsequent histopathological analysis, and cytological examination.

At the Memorial Sloan Kettering Cancer Center, the student performed several activities within the department of Comparative Pathology, under the orientation of Alessandra Piersigilli (DVM, PhD, Dip. European College of Veterinary Pathology), Sebastian Monette (DVM, Dip. American College of Veterinary Pathology) and Adam Michel (DVM, Dip. American College of Veterinary Pathology). The activities ranged from necropsies of several animal species used in research labs such as mice, rats, zebrafishes, non-human primates, ferrets, rabbits, hamsters and pigs; histopathological analysis of both research and diagnostic cases; development and interpretation of phenotyping studies. The student also presented two pathology cases at the weekly Joint Pathology Rounds with diplomate pathologists and residents of the American

College of Laboratory Animal Medicine. During the internship, the student strengthened his knowledge on laboratory animal pathology, comparative medicine and infectious disease pathology. Special focus was given in research projects regarding infectious diseases such as Clostridium difficile and Mycobacterium Tuberculosis in mice and rats and phenotyping studies of mice models of vascular malformations and degenerative neuromyopathies, among others.

Overall, the internship year was crucial and allowed the strengthening of competences and knowledge in veterinary pathology, specialty in comparative and laboratory animal pathology, therefore enhancing autonomy under the microscope; and development of communication skills between technicians and investigators.

CHAPTER II: Revision

1. Introduction: Aging

The aging process is a ubiquitous and complex phenomenon defined as a time-dependent functional decline, progressive loss of physiological integrity and progressive increase in disease susceptibly (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Franceschi & Campisi, 2014; Mitchell, Scheibye-Knudsen, Longo, & de Cabo, 2015). The main cause of aging is the accumulation of cellular damage that increases the risk of development of several diseases such as cancer, diabetes, cardiovascular disorders, neurodegenerative disorders, kidney disease, immune decline, among others (López-Otín et al., 2013; De Magalhães 2013; Gems & Partridge, 2013; Kaeberlein, 2016). Age is hence the main risk factor for the most prevalent diseases in developed countries (Niccoli & Partridge, 2012). As living organisms age, cellular and tissue degeneration and atrophy, cellular senescence and dysregulation, neoplasia, chronic inflammation, and tissue repair and scarring occur and may manifest as diseases (Ward, Youssef, & Treuting, 2016). In our society, aging has become an increasing socioeconomic problem all over the world and the older population is increasing over the years (fig. 1). According to the United Nations, the number of older persons is growing faster than the number of people in all younger age groups; and the global population aged 60 years or over was numbered 962 million in 2017, more than twice as large as in 1980 where the number of older people was 382 million. This number is continually increasing, being expected to be 2.1 billion by 2050 (United Nations, 2017). In the European Union the number of people over 75 years is projected to double by 2060, compromising 20% of the population, demanding an increase in 20 to 40% in the investment of healthcare services (Santulli et al., 2015).

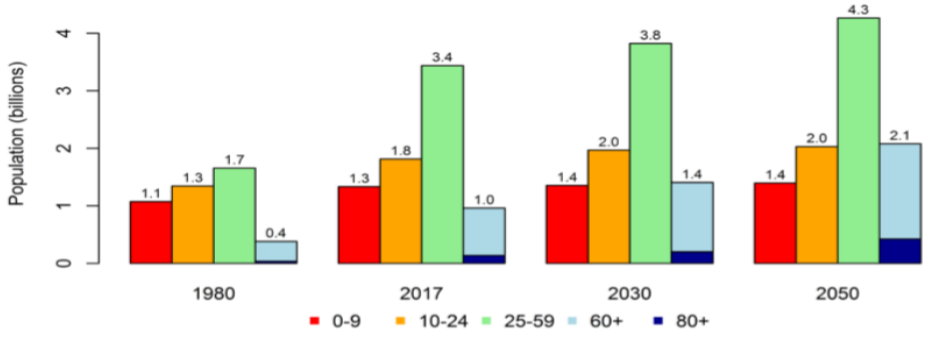


Figure 1) Global population by broad age group, in 1980, 2017, 2030 and 2050. Currently there are approximately 1.0 billion aged individuals. In 2050, the number of aged individuals is more than double that in 2017. Data source: United Nations (2017). World Population Prospects: the 2017 Revision

This demographic phenomenon will lead to a higher incidence of several chronic diseases and will impose a significant economic challenge, thus enhancing the importance of aging research (Harkema, Youssef, & de Bruin, 2016).

1.1 Cellular and molecular alterations in aging

Over the last years, research in the field of aging has aimed to understand the molecular and biochemical mechanisms that cause living organisms to decline in function over time therefore leading to an increased risk of morbidity and mortality (Martin, 2001; Kaeberlein XY, 2016). By delaying the senescence process, it may be possible to reduce the impact of age-related diseases, leading to great benefits for society and the quality of life (Bitto et al., 2016).

In 2013, López-Otín *et al.* presented nine hallmarks that are considered to contribute to the aging process. These are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostatis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication.

Genomic instability encompasses DNA alterations such as point mutations, deletions, insertions, chromosomal rearrangements and chromosomic numerical changes (Vijg & Montagna, 2017). Both the stability and integrity of the nuclear and mitochondrial DNA are susceptible to exogenous physical, chemical and biological hazards and endogenous factors such as DNA replication errors and reactive oxygen species. With aging, somatic cells accumulate mutations through life, gradually losing genetic stability (Hoeijmakers, 2009; Vijg & Montagna, 2017). Although most DNA damage is repaired, some persists and accumulates over time. Those genetic alterations can originate dysfunctional cells that should normally be eliminated by apoptosis or senescence to avoid loss of homeostasis and consequences such as cancer and degenerative diseases (López-Otín et al., 2013; Vijg & Montagna, 2017). Although DNA damage occurs randomly in the genome, telomeres are particularly susceptible to aging (López-Otín et al., 2013). Telomeres are the DNA–protein complexes at chromosome ends that protect genome from degradation and interchromosomal fusion. In vertebrates they consist of tandem repeats of the TTAGGG sequence (Shammas, 2012; Varela, Lorente, Tejera, Ortega, & Blasco, 2016). DNA polymerases lack the ability to replicate the telomeral DNA therefore in each cell division there is a progressive loss of telomere protective sequences in the chromosomal ends (telomeres shortening), leading to telomere exhaustion (López-Otín et al., 2013; Maestroni, Matmati, & Coulon, 2017) (fig.2). Most somatic cells do not express telomerase, an enzyme that can elongate the telomeres, therefore they will only be capable of a limited number of cell divisions before telomeral length decreases to a critical limit and DNA gets compromised (Aunan, Cho, & Søreide, 2017). When telomeres become critically shortened

the end of chromosomes cannot be protected. Persistent DNA damage, genomic instability and interchromosomal fusion occur, ultimately leading to signaling cell cycle permanently arrests via cellular senescence or apoptosis (Fumagalli et al., 2013; Maestroni et al., 2017; Shay, 2017). Therefore, as most somatic cells age, their telomeres become shorter and they exit the cell cycle, resulting in an inability to generate new cells to replace damaged ones (Kumar, Abbas, Aster, 2015). Short telomeres increase the risk of several diseases such as coronary heart disease, heart failure, diabetes, cancer and osteoporosis (Shammas, 2012). Genetically modified animals established links between telomere loss, cellular senescence and aging. Mice with shortened telomeres have decreased lifespans in contrast with mice with lengthened telomeres, which have increased lifespan (López-Otín et al., 2013; Kumar, Abbas, Aster, 2015).

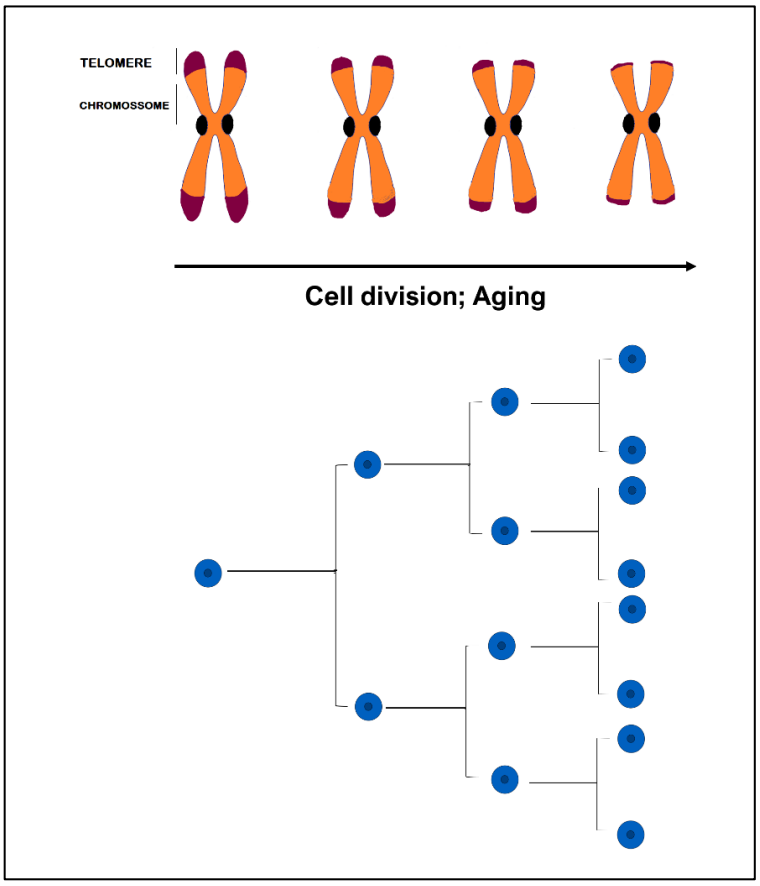


Figure 2)Telomere length in cell division. As cells divide, telomere length becomes shorter. When their length reaches a critical limit DNA damage and interchromosomal fusion frequently occur and cells enter senescence (original)

Epigenetic alterations also have a major impact in aging. Epigenetics refers to the reversible heritable changes in gene expression that occur without any alteration of the DNA sequence. These encompass DNA methylation, histone modifications and chromatin remodeling (López-Otín et al., 2013; Pal & Tyler, 2016). Chromatin remodeling is crucial to cellular process such as transcription and DNA repair and is coordinated by histone-modifying enzymes and ATP dependent chromatin remodeling complexes. Defects in chromatin remodeling are associated

with pathology during aging (B. Liu, K.H Yip, & Zhou, 2012). There is also a progressive hypomethylation of the majority of the cellular DNA with aging, except for some specific genes, including several tumor suppressor genes, that become hypermethylated with age (López-Otín et al., 2013; Pal & Tyler, 2016).

With aging, the ability of cells and tissues to preserve proteostasis is gradually compromised leading to several age-related diseases both animals and humans, such as Alzheimer's and Parkinson's disease (Kaushik & Cuervo, 2015). Proteostasis englobes complex mechanisms that ensure the stabilization of folded proteins like the chaperone system, but also the degradation of proteins like the proteasome and the lysosome-autophagy systems (López-Otín et al., 2013). For instance, the chaperone system is composed by specific proteins whose function is to assist other proteins through conformational changes and *de novo* folding. It is significantly impaired with aging leading to the accumulation of misfolded proteins and therefore compromising tissue homeostasis (Kaushik & Cuervo, 2015; Klaips, Jayaraj, & Hartl, 2018). Once proteins are terminally misfolded they are degraded by the proteasome and the lysosome-autophagy systems, whose function also decline both in humans and animal models of aging (López-Otín et al., 2013; Pyo et al., 2013).

Nutrient sensing is also impaired with aging. In mammals, the somatotrophic axis includes the growth hormone (GH), which is produced in the anterior pituitary leading to the production of insulin-like growing factor 1 (IGF-1) by the cell, which senses the presence of glucose and therefore promotes anabolism, cell growth and replication (Xiwei Zheng, Cong Bi, Marissa Brooks, 2015). The decreased activity of the somatotrophic axis increases longevity and lifespan in animal models (López-Otín et al., 2013). Among several receptors and mediators of nutrient sensing, the mammalian target of rapamycin (mTOR) signaling system plays a major role and has attracted special attention by aging researchers. The mTOR is a protein kinase that regulates physiological homeostasis by coordinating anabolic and catabolic processes and is activated by nutrients, energy, oxygen levels, GH, among other substances (Weichhart, 2018). mTOR inhibition increases lifespan and delays several age-related diseases via several mechanisms such as interfering with pathway of IGF-1 and the somatotrophic axis, leading to lower rates of cell growth and metabolism and therefore reduced cellular damage (Kumar, Abbas, Aster, 2015). mTOR inhibition also enhances autophagy therefore providing substrates for energy production under conditions of limited nutrient supply and removal of damaged organelles, reducing the risk of disease (López-Otín et al., 2013; Weichhart, 2018). Rapamycin is a Food and Drug Administration (FDA) approved drug that inhibits mTOR and it's proved to increase lifespan and decrease age-related diseases such as cancer and Alzheimer's in rodent

models of aging. These effects make it a potential drug for increase human longevity and quality of life. mTOR inhibition may also preserve stem cell function, stimulation of the immune system and of mitochondria, decreased cellular senescence (S. Xu, Cai, & Wei, 2014; Kaeberlein, 2016; Weichhart, 2018). Cellular senescence is defined as a stable arrest of the cell cycle accompanied by other phenotypic alterations such as chromatin remodeling, metabolic reprogramming, increased autophagy and the secretion of proinflammatory mediators known as the senescence secretome (Kuilman, Michaloglou, Mooi, & Peeper, 2010; Mchugh & Gil, 2018). Senescence is triggered in response to cellular damage and stress and it can be induced in normal and aged cells by genetic damage, telomere erosion, epigenetic factors and mitochondrial dysfunction (fig.3) (López-Otín et al., 2013; Mchugh & Gil, 2018).

Senescence is strictly associated with aging. On one hand, it has advantages to the organism because it induces the permanent arrest in the cell cycle. Such blockade ensures that damaged and aged cells do not replicate and perpetuate their genome, therefore limiting tumor onset, progression and maintain homeostasis (Mchugh & Gil, 2018). On the other hand, senescence leads to stem cell exhaustion and is associated with several age-related disorders, typically degenerative, such as glomerulosclerosis, osteoarthritis, atherosclerosis, among others. The senescence-associated secretory phenotype (SASP) consists of proinflammatory cytokines, chemokines, proteases and grown factors, and may be responsible for tissue dysfunction and chronic inflammation observed in senile phenotypes (Franceschi & Campisi, 2014; Malaquin, Martinez, & Rodier, 2016; Mchugh & Gil, 2018).

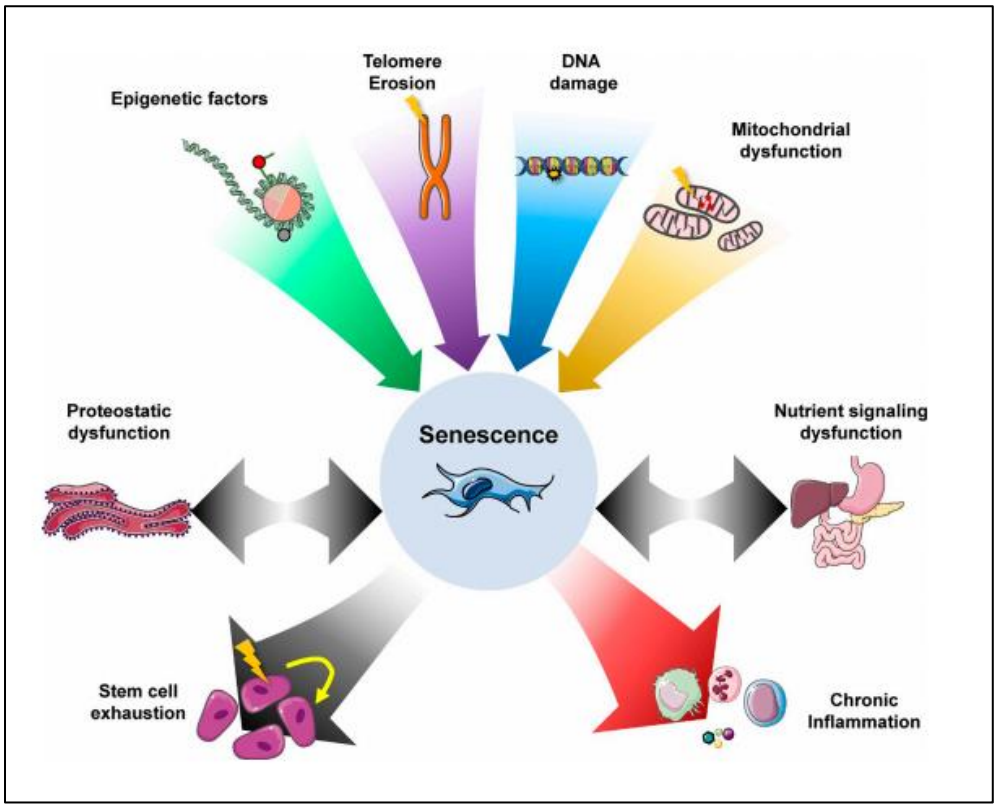


Figure 3) Cellular senescence as the central hallmark in aging. As cells age, epigenetic alterations, telomere shortening, DNA damage and mitochondrial dysfunction can induce a senescence state, leading to stem cell exhaustion, impaired nutrient signaling and proteostatic dysfunction and a persistent chronic inflammation status (data source: McHugh & Gil, 2018)

Stem cell exhaustion is also recognized has a hallmark of aging and is triggered not only by cellular senescence but also by DNA mutations, telomere shortening and epigenetic alterations (López-Otín et al., 2013). As stem cells age, their renewal capacity diminishes and their ability to differentiate into various cell types (plasticity) tends to decrease (Shufian et al., 2017). For example, healing of fractures in older people is slower than in young individuals, hematopoiesis decreases as individuals become old (Chung & Park, 2017; Shufian et al., 2017). There is also evidence that supports the hypothesis that some neurodegenerative disorders are due to the decreased proliferation and increased senescence of neural stem cells (Schultz & Sinclair, 2016). Stem cell function can also be compromised by the hormonal and metabolic changes, as well as the proinflammatory microenvironment, both typical of aging organisms and tissues (Rudolph K.L., 2015; Shufian et al., 2017). This proinflammatory microenvironment is characterized by the increased levels of cytokines such as interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and C-reactive protein. Adaptive immunity declines due to immunosenescence, but innate immunity tends to be activated. In fact, the term "inflammaging" is given to the low-grade, chronic, consistent inflammatory status seen in old individuals in the absence of infection (Franceschi & Campisi, 2014). In normal conditions, inflammatory responses are dampened when proinflammatory factors in infection and tissue damage are

eliminated whereas in aging, the persistent and low proinflammatory microenvironment leads to a chronic flogistic status that becomes harmful to the organism, therefore resulting in tissue damage (Prattichizzo et al., 2016). Inflammaging increases morbidity and mortality and reduces life quality. In fact, there is strong data suggesting the relationship between this inflammatory status and degenerative diseases such as Alzheimer's and Parkinson's diseases, cancer, atherosclerosis, multiple sclerosis, among others (Prattichizzo et al., 2016). Other types of intercellular communication are altered in aging tissues, such as endocrine, neuroendocrine and neuronal communications. For instance, the renin-angiotensin system tends to be deregulated in aging as the proinflammatory microenvironment increases (López-Otín et al., 2013). Also, extracellular adenosine concentration tends to increase in aging as a result of metabolic stress and cell damage (Haskó & Pacher, 2008).

As we can perceive, there is no single cause for the cellular alterations seen in aged tissues, as it is rather caused by a combination of multiple factors leading to the accumulation of DNA, mitochondrial, and protein damage, and impairment in the communication with other cells.

1.2 Aging and cancer

Cancer is a name given to a collection of different diseases characterized by abnormal cell growth (American Cancer Society, 2017). At first glance, cancer and aging appear to be opposite processes; while cancer cells are characterized by an aberrant gain of function and fitness, aging cells are characterized by a loss of fitness (López-Otín et al., 2013; Aunan et al., 2017). However, in a deeper analysis, this two apparently opposite processes share several common characteristics and hallmarks (Aunan et al., 2017).

For example, cancer occurs when normal cells accumulate genomic instability over a period of time and acquire the capability of replicative immortality (Jafri, Ansari, Alqahtani, & Shay, 2016). Genomic instability, a common hallmark of aging, is observed in almost every human cancer. The mutation rate has to be higher than normal, predisposing the cell to malignant transformation (Yao & Dai, 2014). Telomere shortening is also common to aging and cancer (Aunan et al., 2017). Aging cells frequently have short telomeres that elicit DNA damage responses that trigger cellular senescence or apoptosis. In cancer cells however, telomerase upregulation and reactivation is a critical feature in 90% of neoplasms (Jafri et al., 2016). Human telomerase reverse transcriptase enzyme can build back on the telomeral cap ends that otherwise get shortened by each cell division. By avoiding an ever shorter telomeral cap end promoted by telomerase, cancer cells can bypass cellular senescence and apoptosis and continue to proliferate (Aunan et al., 2017).

Epigenetic changes are also observed in cancer cells. Aging cells are characterized by a global hypomethylation, except for tumor suppressor genes that are hypermethylated (López-Otín et al., 2013). Neoplastic cells show aberrant methylation patterns and are broadly characterized by hypermethylation and silencing of tumor suppressor genes in association with hypomethylation and activation of protooncogenes, resulting in chromosomal instability therefore enhancing tumor growth (Aunan et al., 2017). Post-translational histone modifications also play an important role in cancer biology and in cellular aging (Chervona & Costa, 2012; López-Otín et al., 2013).

Like in aging, cancer cells also show impaired proteostasis. The chaperone system is also implicated in oncogenesis as it regulates proliferation, invasiveness, angiogenesis, and metastasis (Aunan et al., 2017). However, while its activity is reduced in aged tissues, over-activity is observed in cancer cells. A similar trend is seen also for autophagy. Autophagy has a dual role: on one side it promotes tumor growth, cell survival during chemotherapy and blockadge of apoptosis, but on the other side it induces tumor suppression by controlling cell growth and angiogenesis (Mathew, Karantza-Wadsworth, & White, 2007; Aunan et al., 2017).

Nutrient sensing is deregulated both in cancer and aged cells. mTOR pathway plays a critical role in cellular metabolism, growth and proliferation and its inhibition increases not only lifespan and reduces aged-related diseases but it is also used in treatment in some cancers. mTOR signaling is infact up-regulated in neoplastic cells and it is involved in the development, progression and metastasis of many different tumors such as colorectal, prostate and breast cancer (López-Otín et al., 2013; Aunan et al., 2017; Weichhart, 2018).

Although cellular senescence is the antagonism of cancer, it has also a vital role in oncogenesis. In a way, senescence is an anticancer mechanism as it inhibits the proliferation of cells with telomere attrition and DNA damage (Aunan et al., 2017). However, senescent cells can promote oncogenesis due to the senescence-associated secretory phenotype (SASP), whose components are able to promote tumor invasion and angiogenesis (Campisi, 2014).

In summary, aging and cancer appear to be, despite important differences, two faces of the same coin, which is the time dependent accumulation of cellular damage (López-Otín et al., 2013).

2. Animal models in aging research

The use of animal models to study human anatomy, physiology and pathology has been a major issue since ancient times. The first report of utilization of animal models for human medicine application goes back to the 6th century before Christ (b.C.), when Alcmaeon of Croton determined by studying the brain of dogs, that the brain was the seat of intelligence and sensatory integration. Two centuries later, Aristotle conducted the first studies on embryology by using chickens (Mason, 2009). Beside those examples, some researchers and historians believe that the primordial animal and medical studies were conducted in ancient land of Egypt and were recorded in papyrus at the great Library of Alexandria before it was tragically set on fire in 48 b.C. , resulting in the loss of many years of research (Serageldin, 2013).

By the beginning of the 20th century, the use of animal models increased dramatically and in 1987, 2250 years after Aristotle, Mario Capecchi and his team developed the first knock-out mice. In 2002 researchers from all over the world were able to sequence the whole mouse genome (Mason, 2009).

In the last couple of decades, several animal models have been at the forefront of aging research, leading to remarkable breakthroughs not only in molecular pathways of aging but also in interventions that may increase lifespan (Sprott, 2011). By understanding the relationship between aging and disease, research will allow us to intervene in the aging process and promote a healthy longevity (Kaeberlein, 2016). The best model for studying human aging is, of course, Man. However, ethical issues, enormous life span, genetic heterogeneity and environmental and nutritional statuses limit the type of information one can get from human studies, and therefore model organisms are still the gold standard tool to study aging (Sprott, 2011) (fig.4).

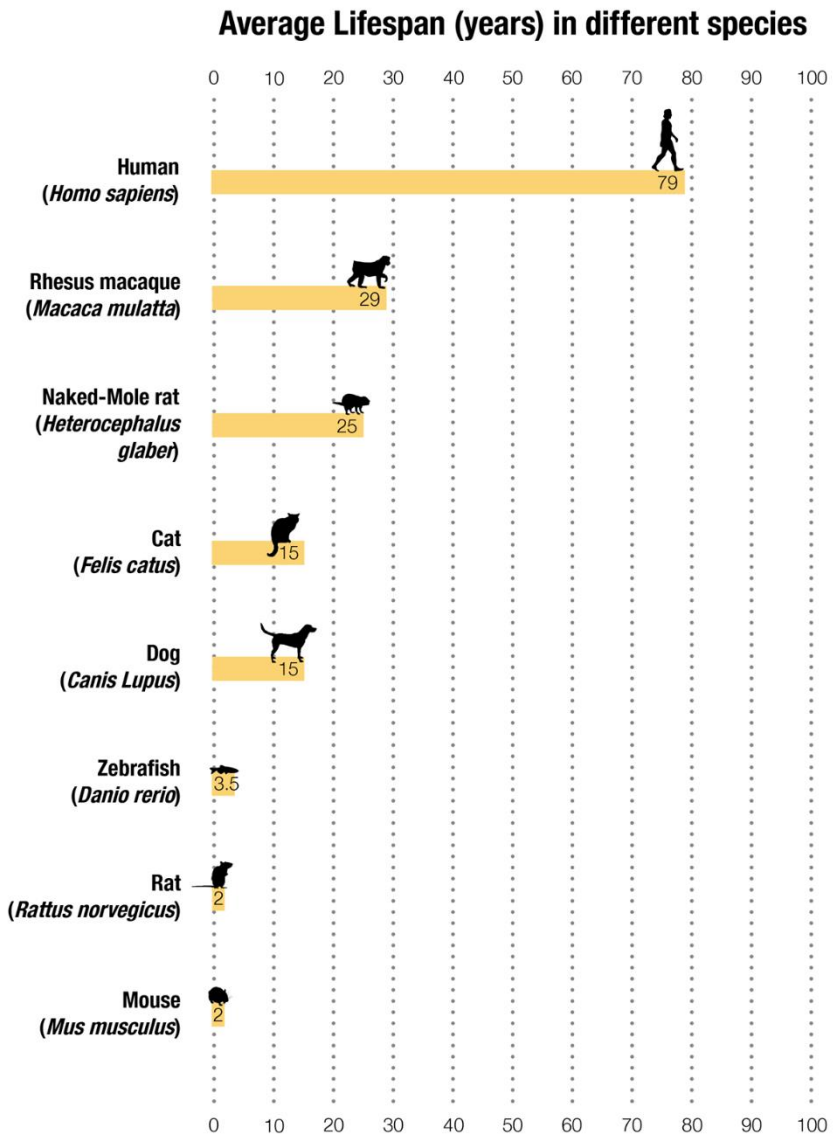


Figure 4) Average lifespan in Humans and animals commonly used for aging research studies (Original)

The first aging studies were conducted in the invertebrate nematode *Caenorhabditis elegans* and on the fly fruit *Drosophila melanogaster*; and were crucial not only to understand the basic molecular mechanisms of aging but also to make us realize that several aspects of the aging process are highly conserved across wide evolutionary distances (López-Otín et al., 2013; Kaeberlein, 2016). In fact, regarding the nine hallmarks of aging previously discussed, telomere attrition is the only feature that has not been associated with aging in invertebrates (Kaeberlein, 2016). Although the comparative genomics between *Caenorhabditis elegans* and of mammals warrants that the first will continue to be a valuable animal model for aging research, the

majority of studies currently conducted all over the world use rodents as the preferential animal model of human aging (AFAR, 2011). Rodents such as rats and mice are the favourite species in aging research, not only because they are mammals and therefore more close to humans than flies and nematodes, but also because their small size and short life span makes them easier to study, compared to long-lived animal such as non-human primates (AFAR, 2011). Also, the background knowledge, ethical considerations, ability to control environmental and nutritional factors, availability of genetic tools and economic issues all contribute to this high relevance of rodent models (Sprott, 2011). For instance, through genetic manipulation researchers have been able to create mouse models of Alzheimer's disease, diabetes, atherosclerosis, progeroid premature aging syndromes, among others (Santulli et al., 2015).

2.1) Mouse (*Mus musculus*)

Mice are the more popular animals used in aging research. Inbred mice, with genetic similarity between different individuals, are usually used by scientists to increase reproducibility of aging studies because, since all the animals share the same genetic background, differences in the results are due to the specific intervention that is being tested (whether it is a specific compound, a genetic modification or an environmental factor) (Sprott, 2011). The big advantage of the diverse inbred mouse strains is that these strains have well-defined and stable phenotypes, described in detail in the literature and databases, such as the Mouse Genome Informatics and the Mouse Phenome Database (Kõks et al., 2016). On the other hand, when using one inbred strain, conclusions cannot be directly extrapolated to other strains or the species, and are not representative of the genetic variability in the human population (Sprott, 2011). Among all the strains, C57BL/6 mice are the most popular among (Kõks et al., 2016), and this strain has specific spontaneous age-related pathologies such as hearing loss, low bone density and susceptibility to obesity, type 2 diabetes and atherosclerosis, that should not be attributed to genetic manipulations or other interventions in test (Jackson Laboratory, 2018).

Genetically engineered mouse models (GEMM) are a powerful tool in aging research. With genetic manipulation at the embryonic stage, researchers are able to upregulate or downregulate the expression of existing genes or to promote the ectopic expression of novel genes in the adult mice. This is possible by the microinjection of a cloned gene of interest into the embryo pronuclei at the one-cell stage (Andersen, 2001). For example, by using a GEMM of a mixed background consisting of 129S1 and C57BL/6Ncr strains with mTOR expression downregulation, researchers were able to conclude that mTOR inhibition increases lifespan and

reduces age-related pathology, thus strengthening deregulated nutrient sensing as an hallmark of aging (Wu et al., 2013).

GEMM are also commonly use as models for accelerated aging and progeria, a human genetic disorder with premature aging phenotypes that leads to a shorter lifespan (Harkema et al., 2016).

2.2) The rat (*Rattus norvegicus*)

Rats are middle-size rodents commonly used by the scientific community for various studies, with special focus on cardiovascular diseases, neurological diseases, renal disease, neuroscience and behaviour studies, cancer and aging (Iannaccone & Jabob, 2009; Sprott, 2011). In the last 30 years, rats started to lose popularity among scientists and a shift in rodent-based research was observed, with the mouse overtaking rat as the model of choice (Ellenbroek & Youn, 2016). This shift was related to availability of techniques for genetic manipulation in mice that started in the late 1980´s (Ellenbroek & Youn, 2016). The first knock-out mouse colony was developed in 1987 and it was only in 2009 that the first knock-out rat was created; and the rat genome was sequenced in 2004, two years later than the mouse genome (Mason, 2009). However, as a model for human physiology and disease, rats offer several advantages over mice as they are closer to humans than mice (Popova, Krivokharchenko, Ganten, & Bader, 2004; Pradhan & Majumdar, 2016). Physiological parameters are easier to measure and more similar to Man, and rats are better animal models for cardiovascular disorders, breast cancer, diabetes and reproductive studies (Iannaccone & Jabob, 2009; Sprott, 2011). Regarding neuroscience, rats have advantages such as the size of the brain, making then better models for surgery, are easier to manipulate and are more affective and easy to tame than mice, making them better models for cognitive studies (Iannaccone, Philip ; Jabob, 2009; Ellenbroek & Youn, 2016). However, although genetic manipulation is possible in rats, an efficient method for the generation of transgenic rat is still a mystery. Pronuclear microinjection technique is primarily used among different methods to generate transgenic rats and involves some challenges as the success rate is very low when compared to mice (Pradhan & Majumdar, 2016). Despite this particular aspect, transgenic rat models of diseases such as Alzheimer, retinal pathologies and neurocognitive models are used in several research labs all over the world (Sprott, 2011; Coelho et al., 2014).

Among different stocks of laboratory rats, inbred Fisher 334 (F334) rats, Lewis rats Brown Norway (BN) rats; F1 hybrids (F334 x BN); and outbred rats such as Wister rats, Sprague Dawley rats and Long-Evans rats are the most popular (Hedrich, 2002; Sprott, 2011). Regarding aging studies, the National Institute on Aging, in the United States of America, uses inbred rats

such as BN and F334 and F334 x BN hybrids (Sprott, 2011). There is fewer information in outbred rats regarding aging pathology, however, Sprague Dawley rats are considered a successful model of cardiovascular and neurocognitive aging (Coelho et al., 2014; Capitanio, Leone, Fania, Torretta, & Gelfi, 2016).

Sprague Dawley is an outbred rat stock commonly used for diabetes, obesity, neurobiology, cancer and cardiovascular diseases. However, the use of such genetically heterogeneous outbred rats may introduce variation in pathology as well as in response to therapeutic intervention (Brower, Grace, Kotz, & Koya, 2015).

2.3 Nonhuman primates

Nonhuman primates are considered the best animal model for the study of aging. This is due to the fact that they share similar physiology and are phylogenetically close to humans, as they share 99% homology with humans (Didier et al., 2016; Mitchell et al., 2015; Simmons, 2016). Among different species, rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fasicularis*) are the most popular nonhuman primates used in aging research , although the improvement of husbandry practices will lead to the expansion of use of smaller nonhuman primates (Didier et al., 2016). Nonhuman primates share similar anatomy, physiology, genetics and cognitive function when compared to humans. Their aggressiveness, intelligence and zoonosis though limit their use in research, both for ethical and health reasons , therefore implying special husbandry (Tardif, Coleman, Hobbs, & Lutz, 2013; Mitchell et al., 2015). Aging research on nonhuman primates focus specially on neurodegenerative diseases such as Alzheimer's and Parkinson's. For instance, rhesus and cynomolgus macaques show neuropathological lesions, like amyloid plaque deposits, with distribution similar to those seen in patients with Alzheimer's (Youssef et al., 2016). Nonhuman primates are also great models for osteoporosis, reproductive decline, immune senescence, chronic inflammation, among other disorders observed in human aging (Didier et al., 2016).

2.4 Naked mole rat (*Heterocephalus glaber)*

The naked mole rat is the longest-lived rodent, with an average lifespan of 25 years and maximum of 30 years (Mitchell et al., 2015). Beyond lifespan, naked mole rats fascinate aging researchers because the physiological decline associated with aging in mammals fail do manifest in these particular rodents (Ruby, Smith, & Buffenstein, 2018). For instance, naked mole rats have highly adapted physiology, high resistance to cancer, rare age-associated chronic

diseases and females do not seem to have menopause, therefore making them a particular good model for aging studies (Delaney, Kinsel, & Treuting, 2016; Ruby et al., 2018). Regarding molecular cell biology, naked mole rats apparently do not meet the classical hallmarks of aging as proteasome function, mitochondrial function and gene and protein expression are maintained with aging (Ruby et al., 2018).

2.5 Non-traditional models of aging

Although rodents and non-human primates are the gold standard animal models for aging research, several other species can be used to understand the pathobiology of aging and can provide valuable input in the study of specific age-related disorders (Mitchell et al., 2015). For instance, dogs and cats are powerful animal model. The most recent demographic studies have concluded that life expectancy in dogs and cats is increasing, and therefore degenerative and age-related disorders will likely increase in the small domestic animal population in the future (Hoffman, Creevy, & Promislow, 2013; Wu et al., 2013), and also, dogs and cats have similar exposure to environmental factors as humans (Mitchell et al., 2015; Youssef et al., 2016). Dogs are also becoming popular for renal diseases because the histological and structural findings in the aging kidney of dogs share several aspects close to the human pathology (Cianciolo, Benali, & Aresu, 2016).

Zebrafishes (*Dario reno*) are also used among researchers. Although their different physiology and their 2 to 3-year lifespan, zebrafish gain attention of the scientific community because of their incredible tissue regeneration capacity and longevity (Gilbert, Zerulla, & Tierney, 2013; Lepilina et al., 2006). They also have human-like short telomeres that progressively decline with age, reaching lengths in old age that are observed in human aging phenotypes (Carneiro, de Castro, & Ferreira, 2016).

3. Phenotyping and genetically engineered rodent models

The word phenotyping consists in assessing observable and measurable characteristics. In genetically engineered models (GEM), phenotyping is usually done to gain information regarding a specific gene by recording clinical, morphological, physiological and cellular changes in rodents resulting from genetic manipulation (Zeiss, Ward, & Allore, 2012; C. Brayton & Treuting, 2018). The final goal of GEM phenotyping studies is the characterization of the measurable effect (morphologic, functional, etc) induced by the genetic manipulation, therefore inferring and understanding the action of that gene in vivo (Zeiss et al., 2012). The

first genetically engineered mouse models (GEMM) were created in the late 1980's, marking the beginning of a new era for biomedical research and a few years later transgenic technologies were also developed for rats (Hanahan, Wagner, & Palmiter, 2007; C. Brayton & Treuting, 2018). Since rodents are relatively inexpensive to maintain, their genomes are mapped, genetic manipulation is doable and share similar physiology to humans, their use as GEM quickly increased allowing the better understanding of specific gene function (C. Brayton & Treuting, 2018). The International Knockout Mouse Consortium has generated more than 16.000 GEM and aims to develop GEM for every protein-coding gene by 2020 (C. F. Brayton, Treuting, & Ward, 2012).

However, the observed phenotypes are due to a combination of genetic factors with environment factors such as diet, housing, microbiome and exposure to pathogens. Thus, genetic variables such as strain, gene-gene interaction and epigenetic factors also contribute to the observed phenotype. When reporting the results of phenotyping studies, veterinary pathologists have to consider the role of both genetic and environment factors before reaching to conclusion regarding the gene function (Dansky et al., 1999; Y. F. Chen, Wu, Kao, & Tsai, 2010; Nishino, Sasaki, Nagasaki, Ahmad, & Agui, 2010; C. Brayton & Treuting, 2018).

3.1 Generation of GEM

In vivo studies of differential gene expression often produce results different from in vitro experiments due to the genetic and physiological complexity of living organisms (Bouabe & Okkenhaug, 2011). There are several different techniques to generate transgenic models both in mice and rats such as transgenic technologies, gene targeting via homologous recombination, conditional mutagenesis, chemical mutagenesis, phage-based homologous recombination systems, among others (O'Sullivan et al., 2006).

With transgenic technologies, exogenous DNA is incorporated into the mouse or rat genome following pronuclear injection therefore allowing gene overexpression phenotype. With this technique, human genes can be introduced in the animal genome by injecting the gene of interest in a newly fertilized zygote pronucleus, and the injected DNA will be randomly integrated into a chromosome therefore allowing gene overexpression (Cho, Haruyama, & Kulkarni, 2009; Bouabe & Okkenhaug, 2011). After transgenesis, the newly genetically engineered zygote is implanted in the animal infundibulum, where it grows and differentiate therefore obtaining transgenic pups (Cho et al., 2009) . After birth the animals are genotyped to determinate the transgenic founders (Cho et al., 2009). Although transgenesis allows the study of human genes and enables *in vivo* characterization of function aspects of the gene of interest, its usefulness in determining the exact role of the gene has some limitations due to the

fact that the exogenous DNA is randomly integrated in the genome, which entails a high variability in copy number of inserted transgenes and the potential interaction of neighbouring genes in the target gene expression and function (Bockamp et al., 2002; O'Sullivan et al., 2006). Other limitation is the low frequency of transgenic founders, ranging from 1 to 5% of genetically engineered zygotes, especially in rats (Hirabayashi et al., 2001). Another alternative for the generation of transgenic rodents is the *Sleeping Beauty* transposon system, which uses plasmid-based DNA transposons for gene delivery by integrating genetic material into the chromosomes of host cells (Katter et al., 2013). This technique is based on the injection into the pronuclei of fertilized oocytes of synthetic mRNA encoding one transposase combined with a circular plasmid carrying the gene of interest, flanked by transposase inverted terminal repeats. Following the delivery of components to the oocyte, transposase is translated and binds the inverted terminal repeats that are flanking the gene of interest therefore catalyzing the excision and subsequent genomic integration of the transposon (Ivics et al., 2014). This technique allows the generation of transgenic animals in frequencies of 50-64, 14-72 and 15% in mice, rats and rabbits, respectively, representing a significant improvement when compared to the pronuclear injection of DNA (Katter et al., 2013).

In contrast, gene targeting via homologous recombination in murine embryonic stem cells (ESC) allows controlled and specific genetic modification by site-specific integration of exogenous DNA into the mouse or rat genome (Bouabe & Okkenhaug, 2011; Gerlai, 2016). The introduction of site-specific modifications into the genome of ESC is achieved by gene targeting through the introduction of a vector and it is the most used technology to generate GEM. The targeting vector is usually composed of three units: 5' homology arm; a positive selectable gene marker which is normally a neomycin resistance gene; and a 3´homology arm (Bouabe & Okkenhaug, 2011). The vector enters the ESC via electroporation and is integrated in the genome by homologous recombination, determinated by the 5' and 3' homology arms and the successful transferred cells are positively selected by culturing the ESC in medium containing neomycin (Bouabe & Okkenhaug, 2011; Gerlai, 2016). This technique is used to generate knock in and knock out animal models by introducing novel genes or producing null mutations, respectively (O'Sullivan et al., 2006; Guan, Ye, Yang, & Gao, 2010). This technique has several advantages when compared with classic transgenics but it also has some disadvantages. For instance, it is limited by availability of correctly positioned restriction sites and requires detailed genetic information regarding the gene of interest in order to design the appropriate targeting vectors. Also it's not tissue or time specific, therefore not allowing temporal and locational control of the gene itself (Cecconi & Meyer, 2000; O'Sullivan et al., 2006). To overcome this obstacle, conditional mutation techniques use a similar but subtler

genetic manipulation strategy that allows not only spatial control of the gene but also organ-specific manipulation by using the Cre/lox recombination system (O'Sullivan et al., 2006; Maizels, 2013). The Cre/lox recombination system is a complex technique commonly used to generate cell-specific gene activation or inactivation by using Cre-recombinase (Song & Palmiter, 2018).

The Cre recombinase is a 38kDA site-specific DNA recombinase from the bacteriophage P1 that recognizes a 34bp site on the bacteriophage P1 genome called loxP and mediates intramolecular and intermolecular site-specific recombination between two loxP sites (Sauer, 1998; O'Sullivan et al., 2006; Song & Palmiter, 2018). The loxP sequence consists of two 13 bp inverted repeats flanking an 8 bp asymmetric sequence that dictates the orientation of the overall loxP site. If the two loxP sequences are in the same orientation, Cre recombinase action will result in the excision of the DNA between them as a covalently closed circle, however, if the two loxP sequences are in different orientation, Cre recombinase will result in inversion of the intervening DNA rather than excision. Besides, if the loxP sites are located on different chromosomes, Cre recombinase mediates chromosomal translocation (Sauer, 1998; Bouabe & Okkenhaug, 2011; Song & Palmiter, 2018). The Cre/lox recombination system allows not only the deletion of genes of interest but also the overexpression of specific genes hence being a powerful toll in genetic manipulation (Bouabe & Okkenhaug, 2011). The loxP sites are inserted in the gene of interest via homologous recombination ("floxed gene") and the conditional expression of Cre recombinase enables to determine in which cell type and when in the animal lifetime the deletion or overexpression of the floxed gene will take place, therefore controlling the gene expression in space and time (O'Sullivan et al., 2006; Bouabe & Okkenhaug, 2011). This is possible through a cell type or tissue specific promoter of the Cre recombinase, by which Cre recombinase is only expressed in certain cells or tissues under a particular promoter (Sauer, 1998; Bouabe & Okkenhaug, 2011).

In recent years, several alternative methods of genetic manipulation in animal models have been developed, with special attention to the Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated 9 (CRISPR/Cas9) system. This system is a complex and powerful tool for genome engineering allowing the rapid production of mutant animals (Teboul, Murray, & Nolan, 2017). Briefly, this technique consists on devising RNA probes that bind not only to the gene of interest but also the Cas9 enzyme. The DNA sequence of that gene will then bind to the RNA/Cas9 complex, which allows the cutting of the DNA by the Cas9 enzyme. Once the DNA is cut, one can use the cell's repair mechanisms to add or delete genetic material or to replace the DNA with a specific customized DNA sequence, allowing gene insertion or

deletion, creating knock in or knock out animals, respectively (Gerlai, 2016; Teboul et al., 2017).

3.2) Generation of transgenic rats

Although the rat is the principal experimental model in biomedical research, in particular in studies of neuroscience, conditional rat transgenic systems are exceptionally rare in this species (Schönig et al., 2012). Although advanced genetic manipulation techniques progressed more in mice, rats are considered to be excellent models for human disease since they have similar physiology to humans rather than mice (Popova et al., 2004). However, mice are predominately used due to the fact that embryo manipulation is very difficult in rats and has several limitations. Mouse embryos are suitable for gene insertion and propagation, in contrast to embryos of rats that are fragile and fail to sustain embryo manipulation for transgenesis (Pradhan & Majumdar, 2016). The success rate after male pronuclear DNA injection in rats is lower than in mice, thus, the majority of rat embryos do not survive after the procedure therefore reducing the efficiency of transgenic rats generation (Charreau, Tesson, Soulillou, Pourcel, & Anegon, 1996).

Transposon-mediated transgenesis is a very useful tool to generate transgenic rats with higher frequency when compared with the standard pronuclear DNA injection and allows the generation of knockout rats (Westlund et al., 2014). However, male pronuclear DNA microinjection using the transposon-mediated transgenesis technology also has it's inconveniences as rats produce high concentrations of transposase enzyme, hence decreasing the transposition phenomenon (Ivics et al., 2014). Another limitation in generating transgenic rats is the difficulty that lies in isolation of embryonic stem cells (ESC). The majority of attempts to isolate ESC in rats were in fact unsuccessful until recent years (Pradhan & Majumdar, 2016). Taken together these challenges associated with transgenic rats' generation led to the decline in the utilization of such species in research. However, in recent years several developments have been made in order to validate transgenesis in rats.

In 2004, Sato and colleges were able to generate a transgenic rat model by using the Cre-Lox recombination system, allowing for the first time the examination of a particular gene function in a temporal and tissue-specific manner (Sato et al., 2004). In 2016, Pradhan & Majumdar developed a noninvasive technique that allows the generation of transgenic rats by injecting the gene of interest in the spermatogonial cells of the testicle, followed by electroporation. This technique revealed to be successful as it skipped the embryo manipulation, therefore reducing the risk of embryonic death (Pradhan & Majumdar, 2016).

Although difficult, ESC have been isolated by several research groups in different strain of rats (P. Li et al., 2009; Tong, Huang, Ashton, Li, & Ying, 2011), genetic manipulation techniques

should be available, optimized and popular among the research community in the near future, allowing the creation of transgenic rats to use as models of human disease based on the establishment of rat ESC, combined with the several advantages of this species over mice (W. Li et al., 2009).

4. Adenosine A_{2A} receptors

4.1 Adenosine

Adenosine is an ubiquitous molecule composed of adenine attached to a ribose, produced primarily from the metabolism of adenosine triphosphate (ATP) that is directly and indirectly involved in key cellular processes (Sheth, Brito, Mukherjea, Rybak, & Ramkumar, 2014; Cunha, 2016). Adenosine can be produced extracellularly and intracellularly, derived from its precursor 5'-adenosine monophosphate (5'-AMP) upon the action of the enzyme 5'-nucleotidase (Sheth et al., 2014). All cells have an intracellular and extracellular receptor-mediated signalling pathway based on adenosine, and therefore this molecule serves as a paracrine signal that coordinate diverse cellular processes across different organs and tissues. Extracellular adenosine regulates several physiological processes such as vasodilation, heart contraction, inflammation, wound healing, angiogenesis, learning and memory (Waarde et al., 2018). Adenosine plays a crucial role in the central nervous system, where it serves as an homeostatic coordinator of brain function (Ribeiro & Sebastião, 2010; Cunha, 2016). Although it is present in low concentration in the extracellular space, adenosine concentrations increase dramatically under stressful metabolic conditions such as inflammation, hypoxia and ischemia, when adenosine concentration may reach the micromolar range (Sachdeva & Gupta, 2013; Waarde et al., 2018).

4.2 Adenosine A_{2A} receptors

The modulatory role of adenosine is mediated by four plasma membrane metabolic receptors from the superfamily of G protein-couple receptors- adenosine A_1, A_{2A}, A_{2B} and A_3- whose binding leads to different transducing signals according to the degree of activation and cellular and sub-cellular localization (Sheth et al., 2014; Cunha, 2016). All of these receptors are broadly expressed not only in the central nervous system but also in other organ systems such as the cardiovascular, immune, pulmonary and renal systems. However differences in the adenosine binding affinity, tissue distribution, expression level and G protein coupling

preference between the different subtypes activates on each of them different signaling pathway (Fredholm, IJzerman, Jacobson, Klotz, & Linden, 2001; Carpenter & Lebon, 2017).

Adenosine A_{2A} receptors are highly expressed in blood cells such as platelets and leucocytes, spleen, thymus and brain areas such as striatum, *nucleus accumbens* and olfactory bulb. It is mildly expressed in the heart, lung, blood vessels and peripheral nerves (Sachdeva & Gupta, 2013; Waarde et al., 2018). Adenosine binding in the A_{2A} receptors activates the G-protein receptor, inducing an increase in adenylyl cyclase activity, which leads to a subsequent rise in intracellular cyclic adenosine monophosphate (cAMP), stimulating cAMP-dependent protein kinase A (PKA) (Sachdeva & Gupta, 2013; Yan, Gao, Cui, Zhang, & Zhou, 2016). As a messenger involved in the regulation of metabolism and biological behavior of cells, cAMP intracellular increase will activate several mechanisms such as cell growth and differentiation, gene transcription and protein expression, therefore initiating several alterations in different organs and systems (Yan et al., 2016).

4.2.1 Adenosine A_{2A} receptors in the hippocampus

Although adenosine A_{2A} receptors have low density in the hippocampus, with predominant presynaptic location, they play an important role in the modulation of synaptic transmission, not restricted to the control of neuronal function but also through their impact on astrocytic and microglial function (Morgado, 2016). Pre-synaptically, A_{2A} receptor activation modulates the release or uptake of glutamate, acetylcholine and GABA (Cunha, 2005). In glutamatergic terminals, these receptors lead to a facilitation of glutamate release and synaptic transmission. Moreover, their activation of Adenylate Cyclase/Protein Kinase A (AC/PKA) pathway has effects on cholinergic and GABAergic transmission, promoting acetylcholine release therefore enhancing GABA transportation into nerve endings (Cristovão-Ferreira et al., 2009; Rebola et al., 2002). At post-synaptic level, A_{2A} receptors interact and modulate the activity of different types of receptors (Ribeiro & Sebastião, 2010). These complex interactions may justify the apparent paradoxical effects of these receptors. In fact, although A_{2A} activation was reported to play a protective role in cases of intracerebral hemorrhage, hippocampal induced excitotoxicity, striatal lesion and spinal cord injury, the inactivation of the same receptor has been proven to afford neuroprotection in contexts of dopaminergic neurodegeneration, β-amyloid aggregation and traumatic brain injury, as well as to reduce cognitive impairment and decrease Aβ levels in the brain of transgenic mouse models of Alzheimer's Disease (Dai et al., 2010). Extracellular adenosine and glutamate levels rise rapidly and dramatically in response to brain insults, due to increased ATP release and altered glutamate presynaptic neuronal release. Higher levels of

adenosine activate preferentially A_{2A} receptors, favoring glutamate release (Cunha, 2005). Thus, this interplay between adenosine and glutamate levels defines the functional outcome of Adenosine A_{2A} receptors, being responsible for a feedforward loop that leads to neurodegeneration (Cunha, 2005).

4.2.2 Adenosine A_{2A} receptors in the immune system

Adenosine A_{2A} receptors are expressed in virtually all cells involved in the inflammatory process and immune system such as macrophages, dendritic cells, mast cells, neutrophils, eosinophils, platelets, fibroblasts, lymphocytes and NK cells (Haskó & Pacher, 2008). There is evidence that A_{2A} receptor stimulation down regulates inflammation by enhancing anti-inflammatory effects, however, several pro-inflammatory effects are also described (Liang et al., 2014). In neutrophils, A_{2A} receptor stimulation leads to a decreased oxidative burst by increasing intracellular cAMP (Linden, 2011). In macrophages, this stimulation induces the inflammatory 1 to macrophage angiogenic 2 like-switch, reduces stimulation by enhancing the inhibition of pro-inflammatory cytokines such as TNF -α and IL-12 and enhances the production of IL-10 and VEGF (Grinberg, Hasko, Wu, & Leibovich, 2009). A_{2A} receptors are also implied in adaptive immunity, specialty by regulating T-cells. Their activation inhibits IL-6 expression while enhancing the production of transforming growth factor β (TGFβ). While IL-6 favors the production of inflammatory Th-17 cells, TGFβ allows the production and protection of anti-inflammatory T regulatory cells, therefore leading to a down-regulation of the inflammatory process (Haskó & Pacher, 2008).

4.2.3 Adenosine A_{2A} receptors in the cardiorespiratory system

In the blood vessels, A_{2A} receptor is important in mediating vasodilation, supporting angiogenesis and protecting tissues from collateral inflammatory damage (de Lera Ruiz, Lim, & Zheng, 2014). In the heart, adenosine A_{2A} receptor stimulation increases contractibility and coronary blood flow by enhancing vasodilatation, improves exercise tolerance and reduces hypertension, preventing cardiac remodeling (Monahan, Sawmiller, Fenton, & Dobson, 2000; da Silva et al., 2017). A_{2A} receptors are also critical for adenosine-mediated protection against ischemia-reperfusion injury in the heart by stimulation and protection of CD4 T-cells that will enhance wound healing and by attenuating the traffic of neutrophils into the infarcted area (Haskó & Pacher, 2008).

This receptors are also broadly expressed in the lung, where they downregulate the activation of pro-inflammatory cytokines such as NF-KB and the expression of NO synthase and protect the lung against acute failure and acute respiratory distress syndrome (Haskó & Pacher, 2008).

4.2.4 Adenosine A_{2A} receptors in the liver

A_{2A} receptors are expressed in several liver cells such as hepatocytes, Kupffer cells and hepatic stellate cells and there is evidence that their stimulation enhances protective effects not only by down-regulating inflammation in general, but also in liver function (Haskó & Pacher, 2008). Ohta and Sitkovsky (2001) showed that KO mice for A_{2A} receptors display an elevated and prolonged production of pro-inflammatory cytokines and severe acute hepatotoxicity after injury, therefore demonstrating the protective effect of these receptors. These receptors also have an important role after liver transplantation, as they are highly expressed by hepatocytes after this procedure in order to suppress the inflammatory response by blocking NF-kB and therefore inflammation in general (Tang et al., 2007). On the other hand, although their suppressive effect in inflammation, adenosine A_{2A} receptors display an important role in the pathophysiology of liver diseases such as fibrosis and cirrhosis, as the stimulation of these receptors in hepatic stellate cells leads to the deposition of collagen in the hepatic parenchyma after injury, therefore enhancing and aggravating cirrhosis (Chang et al., 2006).

4.2.5 Adenosine A_{2A} receptors in the kidney

Adenosine A_{2A} receptors stimulation also display a protective effect on the kidney as they are expressed in mesangial, tubular epithelial cells and renal microvasculature (Haskó & Pacher, 2008). Like the coronary arteries, adenosine stimulation leads to vasodilation of the renal microvasculature, therefore increasing blood flood and enhancing renal protection (Haskó & Pacher, 2008). Their stimulation also has anti-inflammatory effects and reduces the levels of macrophage-derived cytokines such as MCP-1 and TNF-α , both of which are important in the pathophysiology of renal fibrosis and glomerulosclerosis (Awad et al., 2006; Xiao et al., 2013). Unlike in the liver, adenosine stimulation has a protective effect in the development of renal fibrosis. This is probably due to the fact that macrophages are the key elements in the development of fibrosis in the renal parenchyma, unlike in the liver, where stellate cells play a crucial role, and their stimulation with adenosine induces an increase in intracellular cAMP levels, activating the cAMP-PKA-SRC-ERK1/2 pathway inducing the production of type 1 collagen (H. Wang et al., 2014).

4.2.6 Adenosine A_{2A} receptors in cancer and neurodegenerative disorders

Although their protective effect and contribution for homeostasis, adenosine A_{2A} receptors display an important role in several diseases such as cancer and degenerative disorders. Adenosine A_{2A} receptors are highly expressed in several tumor cells such as melanoma, pheochromocytoma, glioblastoma, lymphoma, adenocarcinoma, among others (Mediavilla-Varela et al., 2013; Wardee et al., 2018). Besides tumor cells, their expression is also observed in tumor associated fibroblasts (Mediavilla-Varela et al., 2013). Adenosine stimulation may induce pro-tumor or anti-tumor effects, depending on which adenosine receptor is recruited (Borea, Gessi, Merighi, Vincenzi, & Varani, 2017). The stimulation of adenosine A_{2A} receptors induces several pro-tumor effects such as angiogenesis, cell growth and migration (Gessi et al., 2017). For instance in melanoma cells, when stimulated, A_{2A} receptors increase cell proliferation through several signaling pathways (Gessi et al., 2017). Following tumor growth there is increased tumor cell breakdown and hypoxia and cellular stress, leading to the release of adenosine into the tumor microenvironment. By binding to A_{2A} receptors in immune cells such as T regulatory cells, dendritic cells and tumor-associated macrophages, adenosine induces immunosuppressive effects that also enhance cell growth and tumor proliferation. Therefore, A_{2A} receptors blockade can not only reduce tumor growth but also may counteract the immunosuppressive cloud of adenosine in the tumor microenvironment enhancing tumor cell destruction by immune cells (Leone, Lo, & Powell, 2015).

Besides cancer, adenosine A_{2A} receptor display a role in the pathophysiology of several other diseases, especially in immune disorders and neurodegenerative diseases such as Parkinson, Alzheimer, among others (Franco & Navarro, 2018; Waarde et al., 2018). In immune disorders, an upregulation of these receptors is usually observed, which can be interpreted as a protective measure by limiting the activity of immune cells; however, in other cases such as myasthenia gravis, a downregulation is observed, which could be due to the failure of the protective role and excessive activity of the immune system (Waarde et al., 2018). Regarding neurodegenerative disorders, A_{2A} receptors are up regulated in the brain and glial cells of Alzheimer and Parkinson patients and their blockage reduces the risk of development of these disorders (Franco & Navarro, 2018). A_{2A} receptor blockage may also reduce age related cognitive impairments in animal models and induces protection in mouse models of tauopathy (Batalha et al., 2016; Laurent et al., 2016).

4.2.7 Rat Model of A2AR Overexpression (Tg (CaMKII-hA2AR))

In order to better understand the role of adenosine A_{2A} in the brain, especially in the hippocampus, transgenic SD rats that overexpress human A2AR driven by the CaMKIIα promoter have been generated by Coelho and colleagues (Tg (CaMKII-hA2AR)). Calcium/Calmodulin-activated protein kinase IIα (CaMKIIα) was used has the promoter for neuronal expression for the A2AR gene. CaMKIIα is central to the coordination and execution of calcium signal transduction and is activated by the rise in intracellular calcium concentrations (X. Wang, Zhang, Szábo, & Sun, 2013). High intracellular calcium levels will lead to the binding of calcium to calmodulin. The binding of Calcium/Calmodulin consequently activates a wide range of enzymes including most of Calcium/Calmodulin-activated protein kinases (CaMKs), such as CaMKIIα. CaMKIIα expression is strong and highly heterogeneous, with the dentate gyrus of the hippocampus as the most abundantly expressed region, and its expression is restricted to excitatory neurons such as glutamatergic neurons, and not in GABAergic neurons (X. Wang et al., 2013). Besides, CaMKIIα is only expressed in post-natal neurons, therefore it will not be expressed during the embryonic development stage (X. Wang et al., 2013). Thus, CaMKIIα was selected has the promoter for the A2AR gene, allowing post-natal expression exclusively in glutamatergic neurons (Batalha et al., 2016; Coelho et al., 2014).

The Tg (CaMKII-hA2AR) model exhibits depressive-like behavior, hyper locomotion and impaired exploratory behavior (Coelho et al., 2014). The A_{2A} receptors' overexpression may therefore explain the depression signs that are typically found not only in aging and chronic stress, but also in Alzheimer's disease. The same research group also found that A2AR overexpression in the brain is sufficient to impar the hypothalamus-pituitary-adrenal (HPA) axis and down-regulate glucocorticoid receptors in the hippocampus, which as an inhibitory role in the HPA axis. Therefore, with diminished inhibition from the hippocampus, Tg (CaMKII-hA2AR) rats have higher levels of circulant corticosteroids and chronic stress levels (Batalha et al., 2016). In the present work, we provide an aging pathology study in the Tg (CaMKII-hA2AR) rats in order to characterize the age of onset and incidence of lesion in the heart, lung, blood vessels, liver, kidney, adrenals and other spontaneous lesions such as tumors, in particular mammary fibroadenomas, in order to characterize the influence of the neuronal expression of human A2AR in the aging process.

CHAPTER III- AGING PATHOLOGY IN SPRAGUE DAWLEY RATS: BACKGROUND LESIONS AND COMPARATIVE STUDY BETWEEN WILD TYPE AND TRANSGENIC RATS WITH OVEREXPRESSION OF HUMAN ADENOSINE A_{2A} RECEPTORS

1. Aims and objectives

Previous studies from our collaborators showed that transgenic rats with neuronal overexpression of human Adenosine A2A receptors (Tg (CaMKIIhA2AR)) rats have depressive-like behavior, hyper locomotion, impaired exploratory behavior and impaired hypothalamic-pituitary-adrenal (HPA) axis and, as a result, higher levels of circulant corticosteroids and chronic stress (Coelho et al., 2014; Batalha et al., 2016).

The aim of this work was to evaluate the impact of the neuronal overexpression of human Adenosine A_{2A} receptors in the aging phenotype of major organs and systems, in Sprague Dawley rats. For that purpose, we set two objectives: 1st evaluate and characterize spontaneous lesions in Wild-type (Wt) and Tg (CaMKIIhA2AR) Sprague Dawley rats ranging from 12 to 126 weeks; and 2nd to investigate if those lesions were different, or with different prevalence or incidence between Wt and Tg.

2. Material and methods

2.1) Animals and Husbandry

Our sample consisted on a total of 76 rats (table 1), Among those, 37 Wild-type (Wt) (16 females and 21 males) and 39 were transgenic (Tg (CaMKII-hA2AR)) (18 females and 21 males). The animals were euthanized with anesthetic overdose (isoflurane inhalation) at specific time-points, ranging from 12 to 126 weeks of age, followed by cardiac perfusion (with saline and neutral-buffered formalin) and decapitation. All animal procedures were conducted by personal trained in the appropriate techniques and procedures, according to the national law, the EU Directive and the FELASA guidelines, performed after approval by the Welfare Bodies of of iMM-JLA and by the Portuguese competent authority (Direção Geral de Alimentação e Veterinária). The iMM-JLA Rodent facility complies with the European Directive 2010/63/EC and with the Portuguese Law transposing this Directive (DL 113/2013). Environmental conditions were kept constant, with food and water ad libitum, 21±0.5°C, 60±10% relative humidity and 12 h light/dark cycles.

Animals (n)	**Male (n)**	**Female (n)**
Tg (CaMKII-hA2AR) (39)	21	18
Wt (37)	21	16
Total (76)	**42**	**34**

Table 1) Sample characterization

2.2) Generation of transgenic rats

Transgenic Sprague-Dawley rats overexpressing the human Adenosine$_{2A}$ receptor under the promotor of CaMKII - Tg (CaMKII-hA2AR) – generated as described in Coelho et al. (2014), were used in this study. Briefly, these rats were obtained by microinjection of a linearized DNA construct (fig. 5) into male pronucleus of Sprague-Dawley rat zygotes, consisting on a full-length human A2A cDNA cloned into an expression vector with the 8.5kb mouse CaMKIIα promoter and a polyadenylation cassette of bovine growth hormone. Wt, age and sex-matched Sprague-Dawley rats were used as control**.**

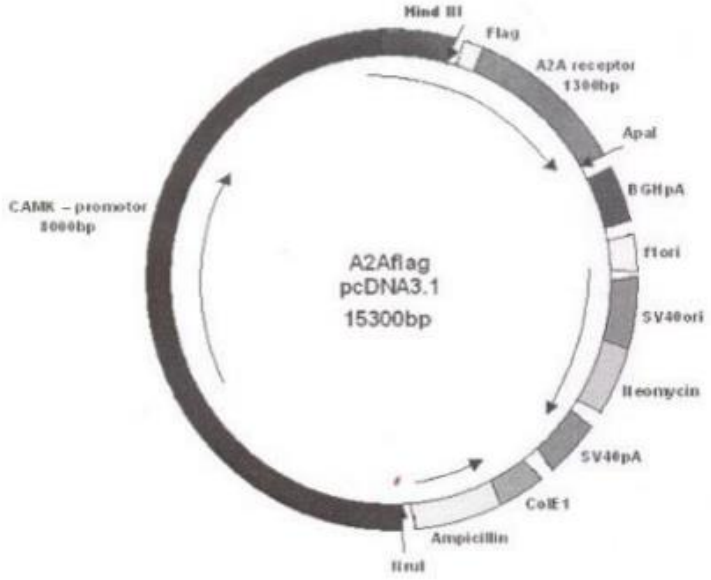


Figure 5) DNA construct used to generate Tg (CaMKII-hA2AR) rats. Construct consists of a full-length human A2A cDNA cloned into an expression vector with the 8.5kb mouse CaMKIIα promoter and a polyadenylation cassette of bovine growth hormone. (Michael Bader, MDC)

2.3) Genotyping

Genotyping was performed by Polymerase Chain Reaction (PCR) (30 cycles, 58ºC annealing temperature) of their genomic DNA isolated from ear biopsies with transgene-specific primers: CaMKII-hA2AR and the housekeeping gene rat β-actin (Invitrogen, table 2)

Primer	Target gene	Organism	Forward primer	Reverse primer	Amplification size
Act-B	β-actin	Rat	AGCCATGTACGTAGCCAT	CTCTCAGCTGTGGTGGTGAA	228 bp
CaMKII-hA$_{2A}$R	Calcium calmodulin dependent Protein Kinase II promoter and human Adenosine A2A Receptor	Transgene	GACTAAGTTTGTTCGCATCCC	GTGACACCACAAAGTAGTTGG	450 bp

Table 2) Primers used for genotyping of transgenic animals

2.4) Histopathology

This was a collaborative study focused on characterizing the histological lesions arising in Sprague Dawley rats that were being used for specific neuroscience/behavioral studies. For this reason, central nervous system was used by our collaborators for *ex vivo* recording and other tests, and not for histopathology.

Necropsy was performed immediately after euthanasia. Macroscopic findings were recorded, and heart, aorta, lung, trachea, left and right kidneys, adrenal glands, liver, mammary gland (in females) and any other organ or tissue with lesions were collected for histopathology. Samples were immersion fixed in 10% neutral buffered formalin, routinely processed for paraffin embedding, sectioned at 4 µm, and stained with hematoxylin and eosin. Von Kossa, Prussian Blue and Masson's Trichrome special stains were performed on selected cases. Lesions were classified according to previously published criteria (INHAND, International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) and scored according to a 5-tier severity scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked. Gastrointestinal system, skin, and reproductive organs were not assessed in this study given the lack of significant histological changes in preliminary studies.

2.5) Statistics analysis

Univariate analysis trough chi-squared and Mantel-Haenszel odds ratio (OR) with 95% confidence interval (95% IC) was used to investigate an association between presence of lesions in the various organs/tissues and genotype. Logistic regression was used for multivariate analysis with 95% IC to further investigate the association between genotype and age. Statistical significance given by p-value (p-value <0.05 was considered significant). Variables coded 0-1 were used for the dependent variable (0, no lesion; 1, lesion), and for the independent variables

gender (0, male; 1, female) and genotype (0, Wt; 1, Tg). Age being a continuous variable was plotted as such. Each organ/tissue was considered as an independent unit. IBM SPSS 25.0 was used for the multivariate analysis (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

3. Results

To investigate the impact of neuronal Adenosine A_{2A} receptor overexpression in the onset of cardiovascular, endocrine, pulmonary and renal pathology in Sprague-Dawley rats, we performed univariate analysis through chi-squared and Mantel-Haenszel odds ratio (OR) with 95% confidence interval (95% IC). As an initial approach, the dependent variable consisted of the number of lesions diagnosed in Wt and Tg rats, regardless of the type of organ/tissue in which they occurred (fig.6). We found that Tg rats are 2.7 times more likely to develop systemic pathology (total of lesions) than Wt rats [OR 2.745, IC 95% 1.0.07-6.997; ($p<0.05$)].

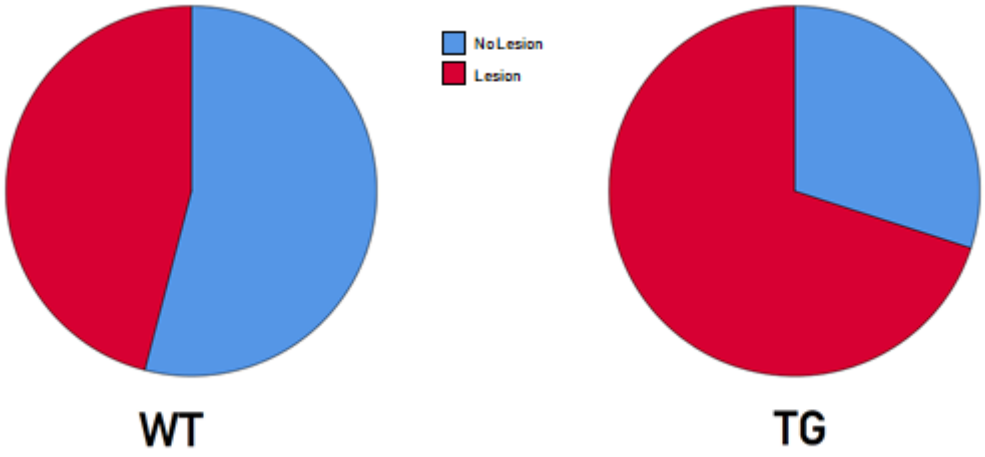
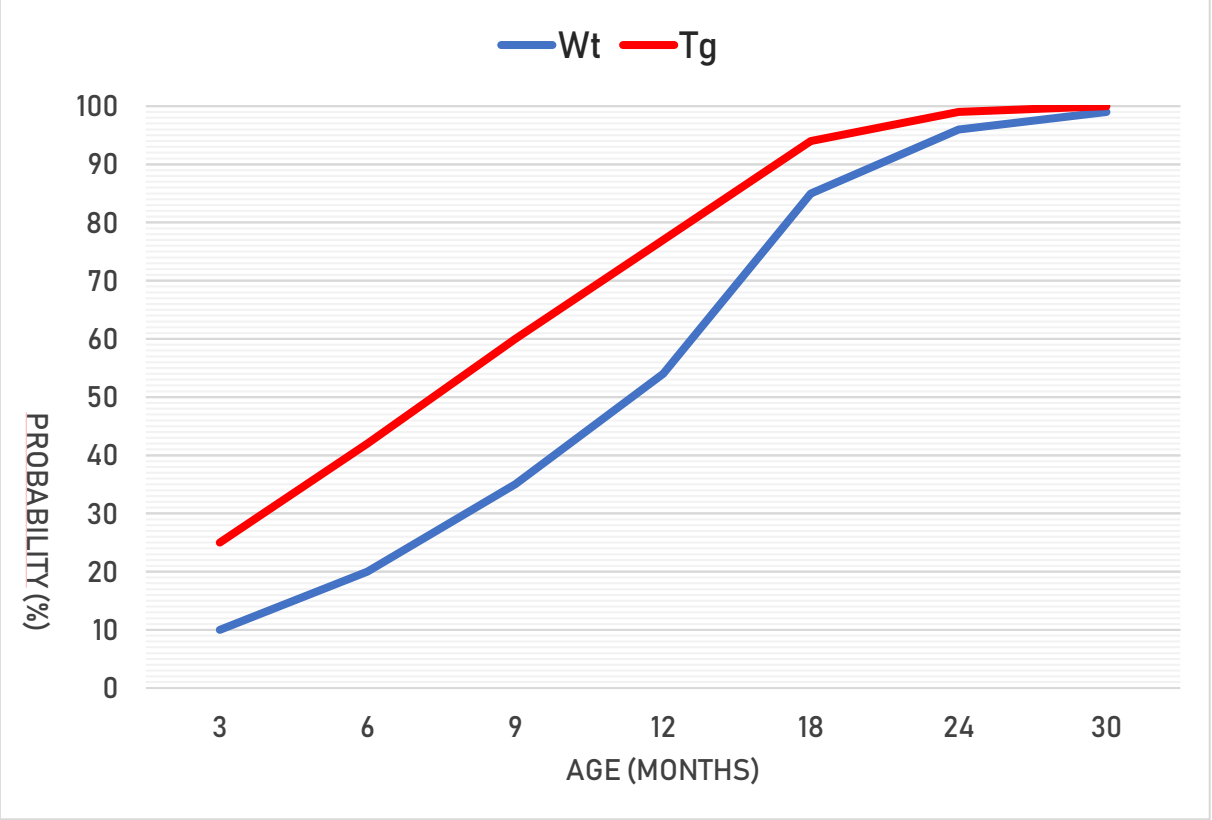


Fig.6) Number of lesions arising in Wt and Tg rats

All lesions found in the animals of our sample, Wt and Tg, were lesions typical of the aging rat. Hence, our next question was whether their occurrence in both genotypes would vary when controlled for age, and to answer this question logistic regression based on age and genotype was performed (multivariate analysis with 95% IC). Our results show that age is the most important factor for the occurrence of pathology, and for every additional week of age the risk of onset of any lesion is increased by 1.060 times [OR 1.060; ($p<0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg are 2.8 times more likely to develop lesions than Wt [OR=2.8; ($p>0.05$)]. In this case however, p value is greater than 0.05 and the null-hypothesis is verified, for which although these results are relevant in our sample, they cannot be extrapolated for the entire rat population. A table with the calculation of the probability for occurrence of pathology in Wt and Tg rats per age group is presented below (Graphic 1).



Graphic 1) Probability for the occurrence of systemic pathology in Wt and Tg rats of increasing age

3.1) Non-proliferative lesions

3.1.1) Cardiovascular

The spectrum, frequency and age of onset of cardiovascular lesions are summarized in Table 3.

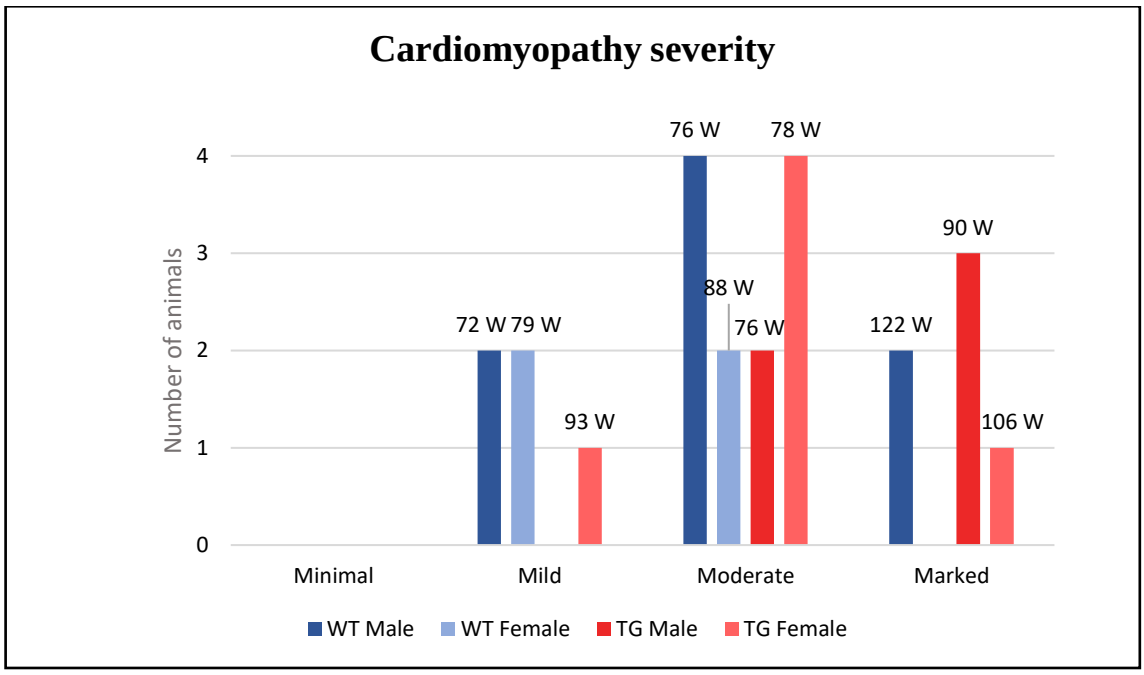
Morphological diagnosis	Wt				Tg			
	Male		Female		Male		Female	
	n	**Age**	**n**	**Age**	**n**	**Age**	**n**	**Age**
Cardiomyopathy	8	78	4	83	5	84	6	85
Degeneration, vacuolar, cardiomyocytes	1	28	4	38	1	32	4	38
Mineralization, medial or intimal, artery	2	56	4	58	9	62	9	87
Atherosclerosis, artery	0	--	0	--	1	77	0	--

Table 3) Spectrum of cardiovascular lesions in Wt and Tg rats. Age (weeks).

3.1.1.1) Heart

Overall, heart lesions consisted on cardiomyopathy, the most prevalent lesion in our sample, and also vacuolar degeneration of cardiomyocytes.

The term cardiomyopathy is used for lesions that encompass a spectrum of morphological changes, including not only cardiomyocyte degeneration but also inflammatory cell infiltration, interstitial proliferation of fibroblasts and connective tissue (fibrosis) (Berridge et al., 2016). These can be focal, multifocal or regionally extensive, hence of different severity, for which they were classified using a score of 1 to 4 (minimal, mild, moderate, marked) (graphic 2) (fig.7).



Graphic 2) Cardiomyopathy score system in Wt and Tg rats (W=weeks).

Mild cardiomyopathy consisted on focal areas of discrete cardiomyocyte degeneration and necrosis, with few infiltrating mononuclear cells and scant amounts of fibrous tissue, while marked lesions consisted of focal to focally extensive or multifocal areas of severe cardiomyocyte degeneration and necrosis, with many infiltrating mononuclear cells and large amounts of interstitial proliferation of fibroblasts and fibrous tissue.

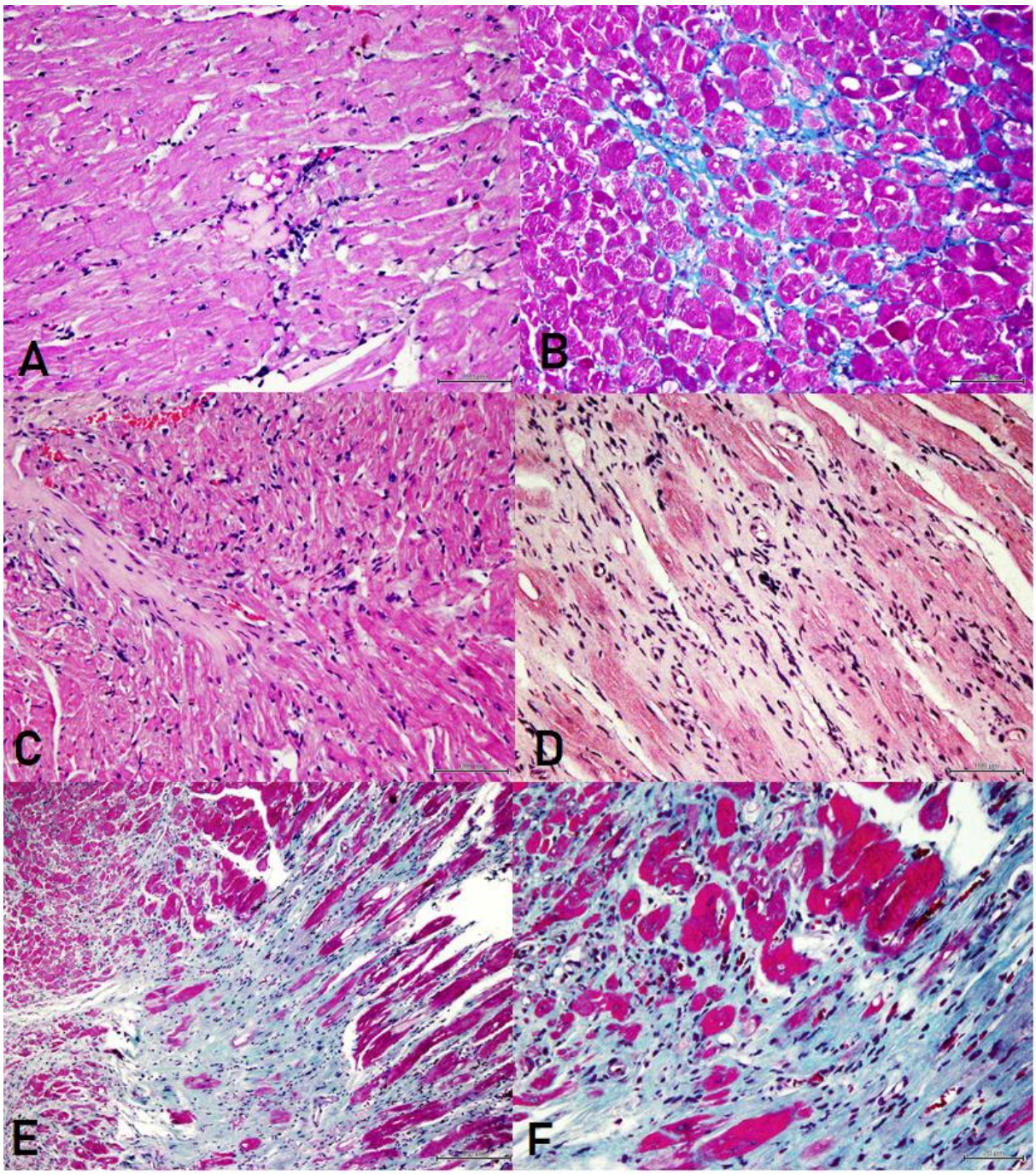
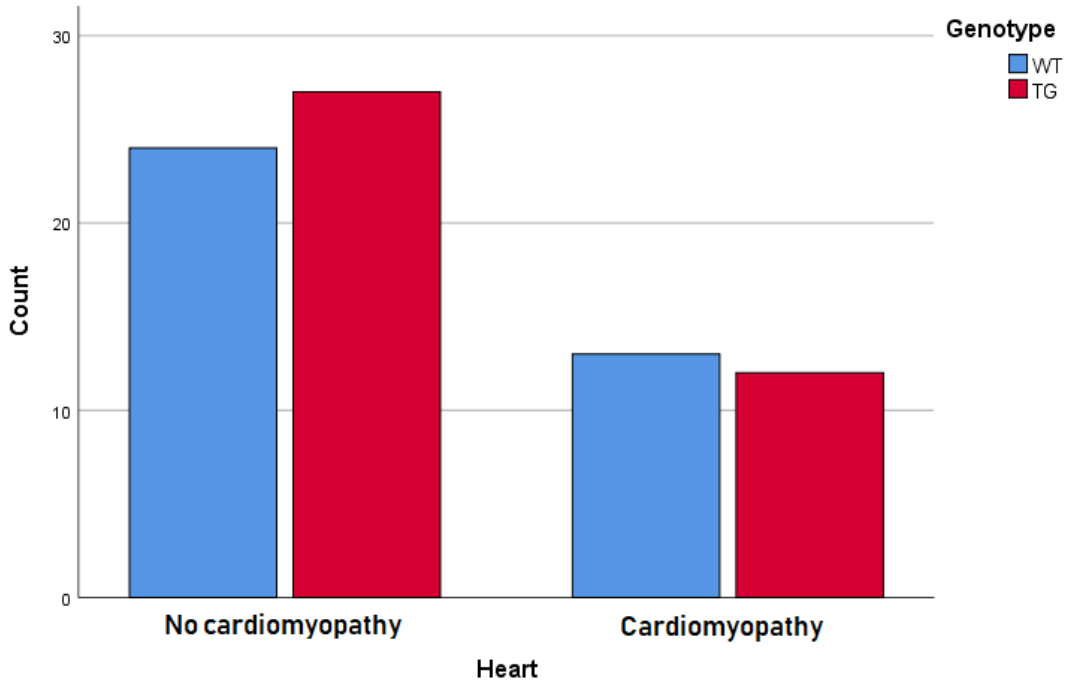


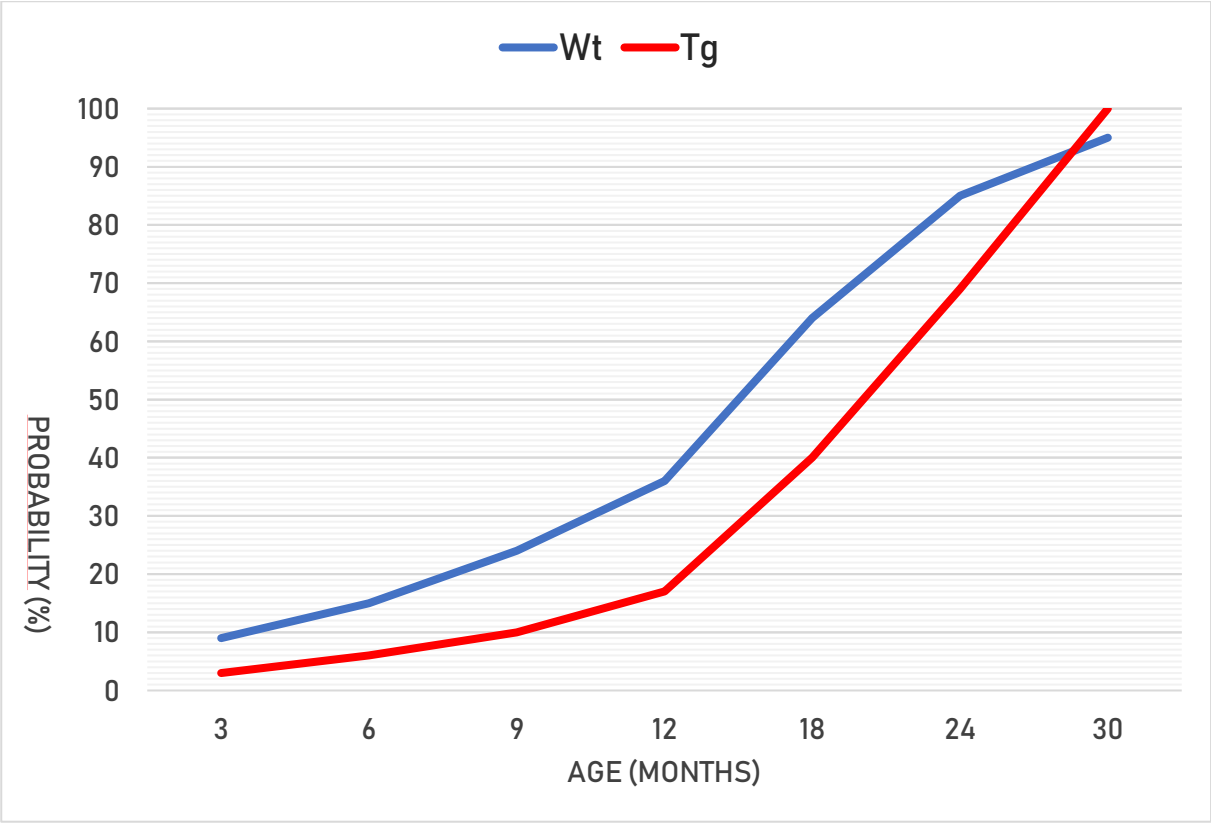
Fig. 7) Cardiomyopathy, heart. A) Mild cardiomyopathy with cardiomyocyte degeneration, mononuclear inflammatory cell infiltration and scant amount of interstitial fibrosis. HE **B)** Area of the myocardium dissected by scant amount of interstitial fibrosis. Masson's Trichrome. **C)** Moderate cardiomyopathy with cardiomyocyte degeneration, mononuclear inflammatory cell infiltration and mild fibrosis. HE. **D)** Marked cardiomyopathy with cardiomyocyte degeneration, mononuclear inflammatory cell infiltration and fibrosis. HE. **E, F)** Enlarged cardiomyocytes in an area of the myocardium dissected by interstitial fibrosis. Masson's Trichrome.

Cardiomyopathy was the most prevalent cardiac lesion in this study, both in Wt (eight males and four females with mean age of 78 and 83 weeks, respectively) and Tg rats (five males and six females with mean age of 84 and 85 weeks, respectively), and its incidence did not differ between groups [OR 0.82; IC 95% 0.315-2.139, (p>0.05)] (graphic 3).



Graphic 3) Cardiomyopathy in Wt and Tg rats.

Logistic regression including age and genotype revealed that age was the only significant risk factor for the development of cardiomyopathy and for every additional week of age the risk of onset of pathology is increased by 1.046 times [OR=1.046; (p<0.05)]. No significant difference was seen between Wt and Tg [OR 0.377; (p>0.05)]. A table with the calculation of the probability for occurrence of cardiomyopathy in Wt and Tg rats per age group is presented below (Graphic 4).



Graphic 4) Probability of occurrence of cardiomyopathy in Wt and Tg

Vacuolar degeneration was the second most common lesion of the heart in both Wt (one male and four females, with mean age of 28 and 38weeks, respectively) and Tg rats (one male and four females, with mean age of 32 and 38 weeks, respectively) and it consists solely of well-circumscribed and discrete areas of microvesicular changes in the cardiomyocytes, with presence of small and clear cytoplasmic vacuoles in the absence of any other degenerative or inflammatory changes (Berridge et al., 2016) (fig.8).

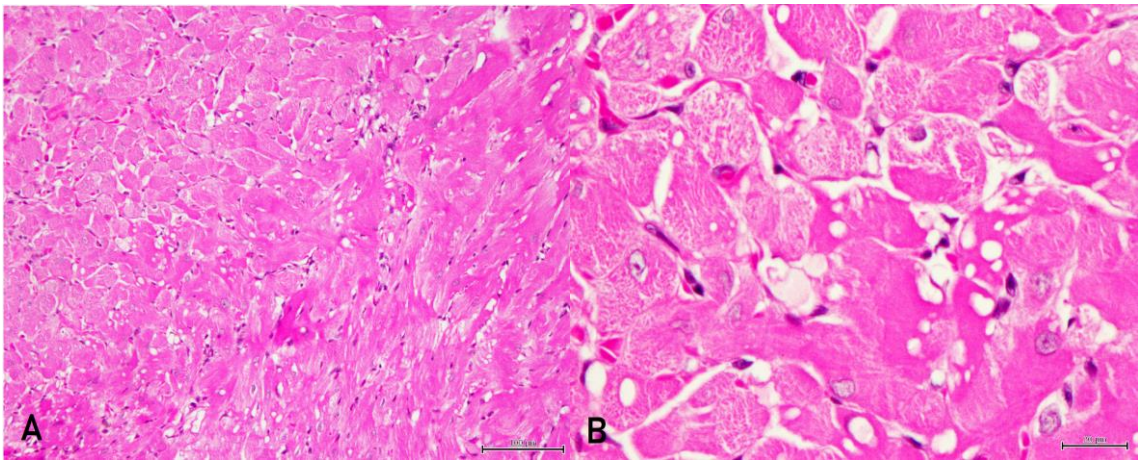


Fig. 8) Degeneration, vacuolar, heart. Microvesicular degeneration of cardiomyocytes. HE

3.1.1.2) Blood vessels

Mineralization was the most prevalent vascular lesion. It consists on the presence of basophilic granular material deposits in the wall (tunica media) of arteries or veins (Berridge at al., 2016) (fig.9). Calcium stain von Kossa was used to confirm that the deposited mineral corresponded to calcium (fig. 9C). This lesion is reported to occur following injury (dystrophic mineralization) or as a primary process following systemic calcium phosphorus imbalance (malignant calcification) and is a common lesion in aging rats (Berridge et al., 2016). Herein they were seen in the aorta, branches of the pulmonary artery (within the lung parenchyma) and renal artery. Often these were associated with cartilaginous foci (fig 9B).

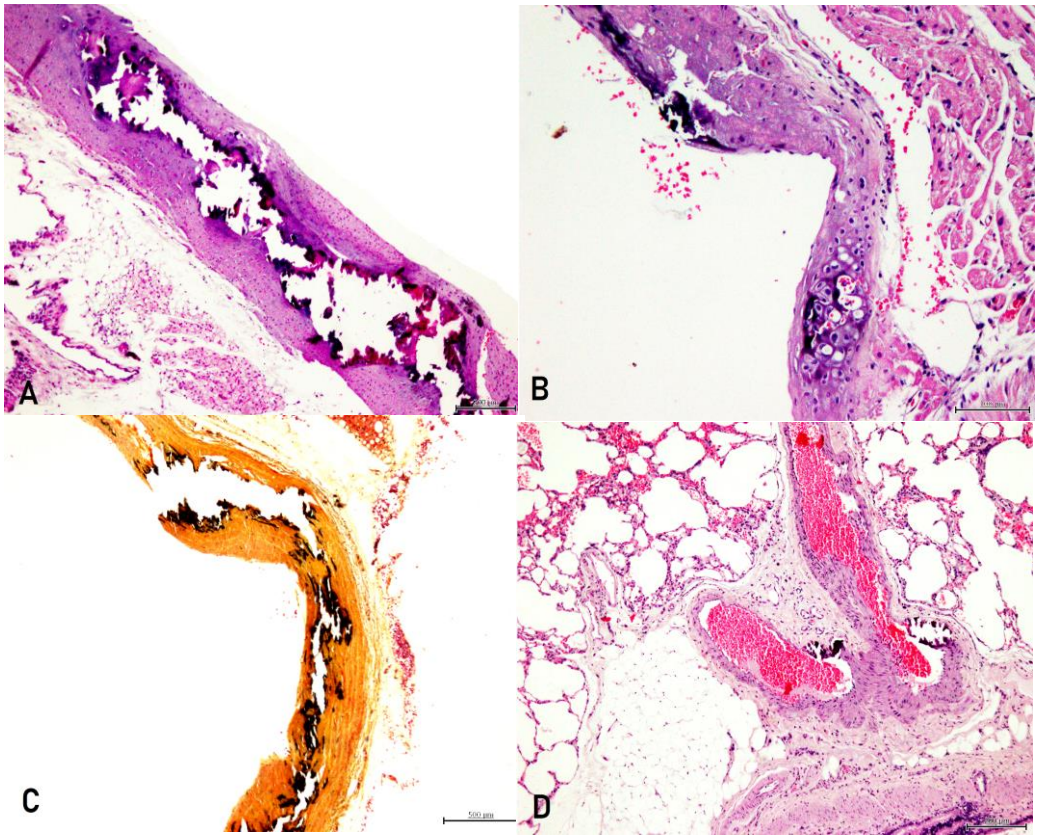
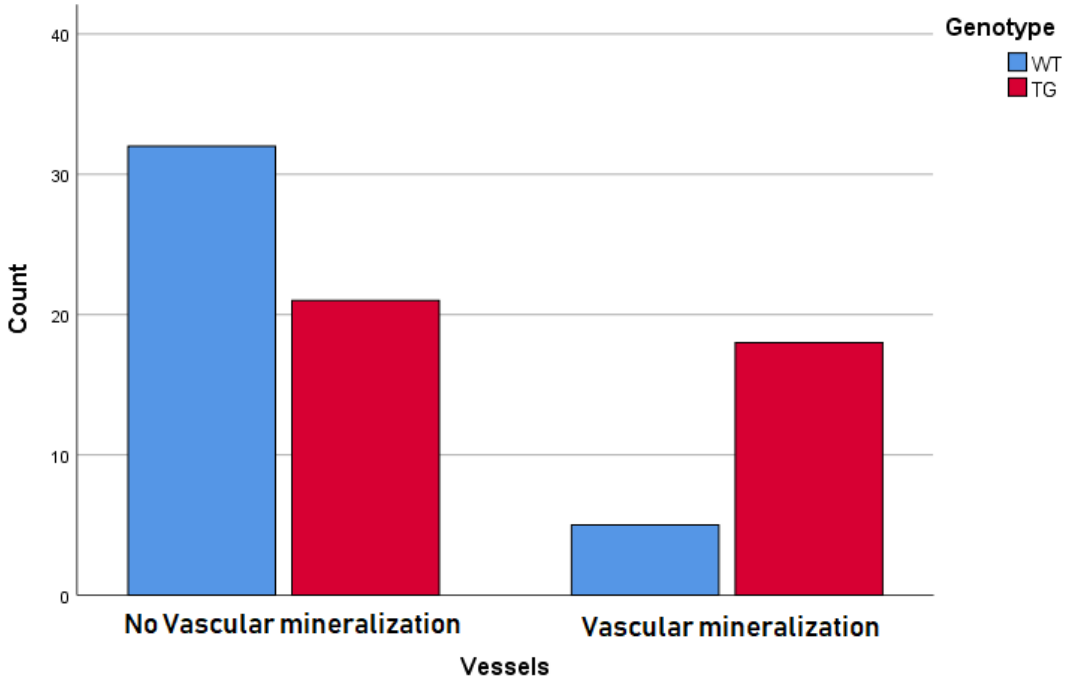


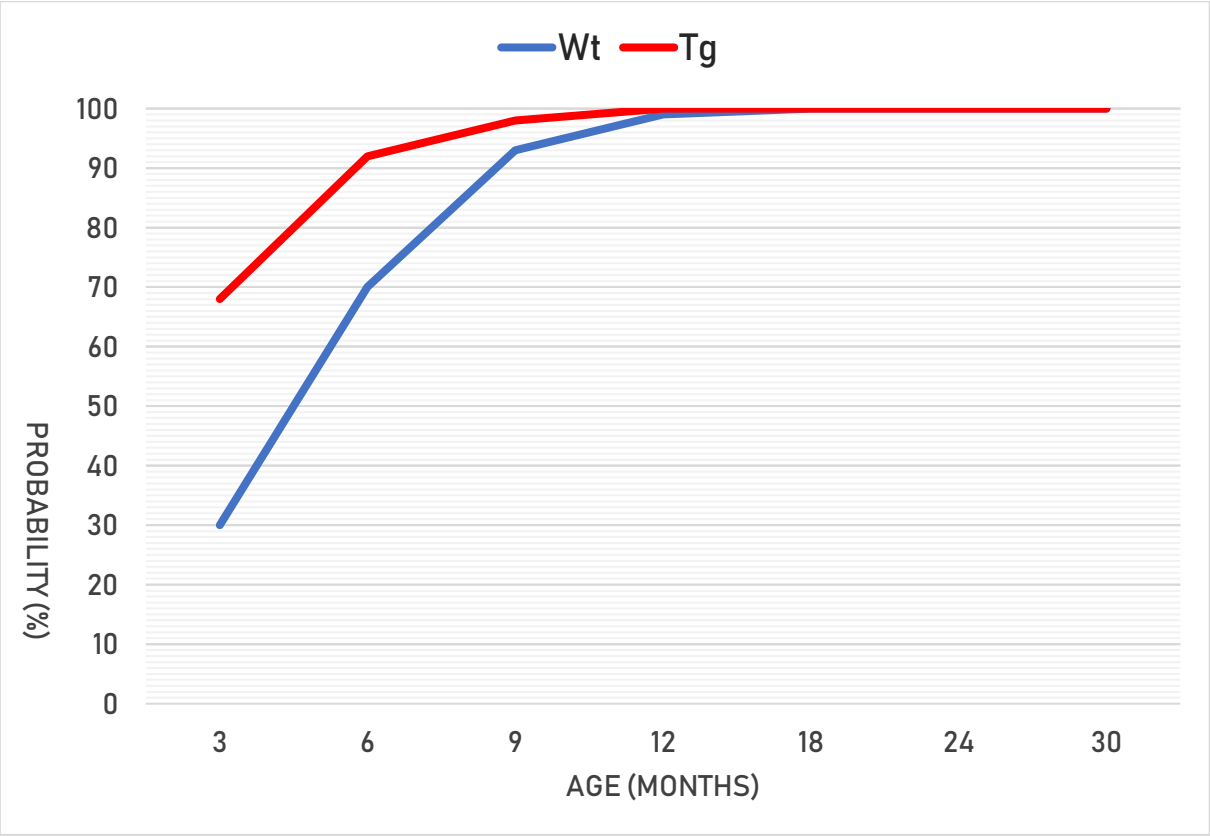
Fig. 9) Mineralization, mural, artery. A) Mineralization of the tunica media of the aorta, HE. **B)** Mineralized aorta with a cartilaginous foci, HE. **C**) Calcium deposits in the tunica media of the aorta, stained in black. von Kossa. **D**) Mineralization foci of the pulmonary arteries, HE.

Mineralization was the most frequent vascular lesion, both in Wt (two males and two females, with mean age of 56 and 58 weeks, respectively) and Tg (nine males and nine females, with mean age of 62 and 87 weeks, respectively). Tg rats are 5.5 times more likely to develop vascular pathology than Wt rats [OR 5.486, IC 95% 1.776- 17.074; ($p<0.05$)] (graphic 5).



Graphic 5) Vascular mineralization in Wt and Tg rats.

Logistic regression including age and genotype revealed that age had a low impact on the development of vascular mineralization [OR 1.013; ($p>0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg are 4.9 times more likely to develop lesions than Wt [OR=4.954; ($p<0.05$)]. A table with the calculation of the probability for occurrence of vascular mineralization in Wt and Tg rats per age group is presented below (Graphic 6).



Graphic 3) Probability of occurrence of vascular mineralization in Wt and Tg rats

One single case of atherosclerosis was observed in a 77-week male Tg rat (fig.10). This lesion encompasses the expansion of the intima in a large artery with a heterogenous matrix of lipid, smooth muscle cells and mononuclear inflammatory cells that can be foamy.

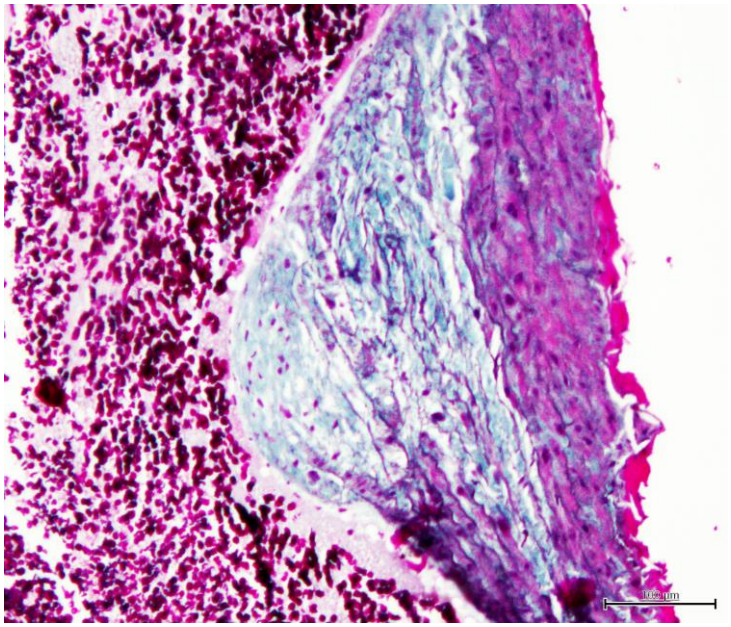


Fig. 10. Atherosclerosis, artery. Expansion of the intima of a large artery. Masson's trichome.

3.1.2) Respiratory system- Lung

The spectrum, frequency and mean age of onset of lung lesions are summarized in Table 4.

Diagnosis	Wt				Tg			
	Male		Female		Male		Female	
	n	Age	n	Age	n	Age	n	Age
Alveolar histiocytosis, terminal bronchioles, alveoli	1	77	1	80	1	75	5	65
Mineralization, terminal bronchioles, alveolar septa	0	--	0	--	3	103	2	89

Table 4) Spectrum of lung lesions in Wt and Tg rats. Age (weeks)

Overall, lung lesions consisted on alveolar histiocytosis and mineralization of the alveolar septa. Alveolar histiocytosis was the most frequent lesion and consists of variable degrees of intra-alveolar aggregation of macrophages containing foamy cytoplasm (Renne et al., 2009)(fig.11).

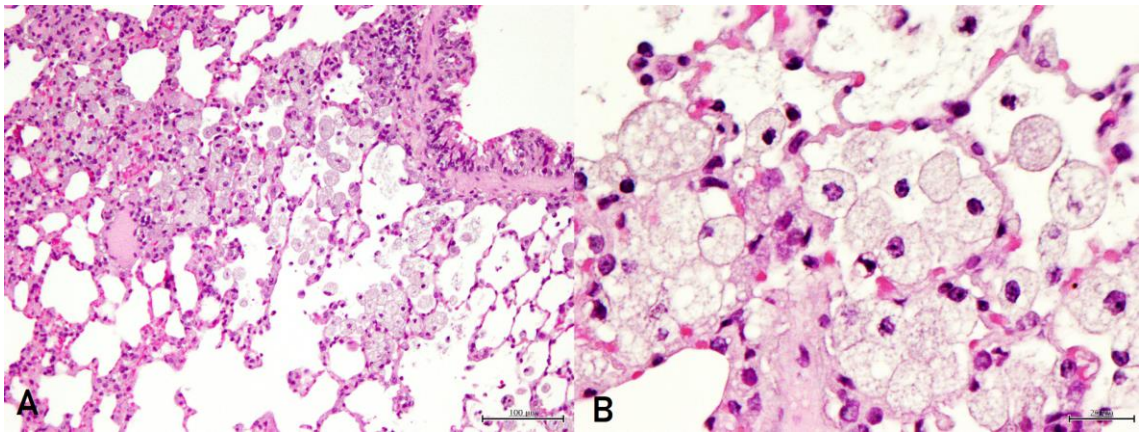


Fig. 11- A, B) Alveolar macrophage aggregation, lung. Aggregates of alveolar macrophages (histiocytosis). HE.

Mineralization of the alveolar septa was the second most diagnosed lesion and consists of linear mineralization of the alveolar septa, usually accompanied by macrophage infiltration , visible on hematoxylin-eosin and confirmed with stains for minerals (von Kossa) (Renne et al., 2009) (fig.12).

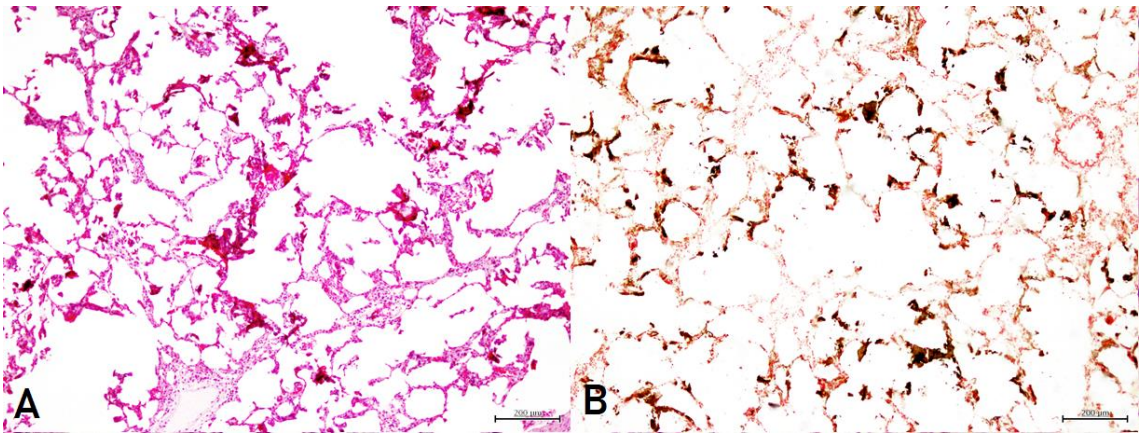
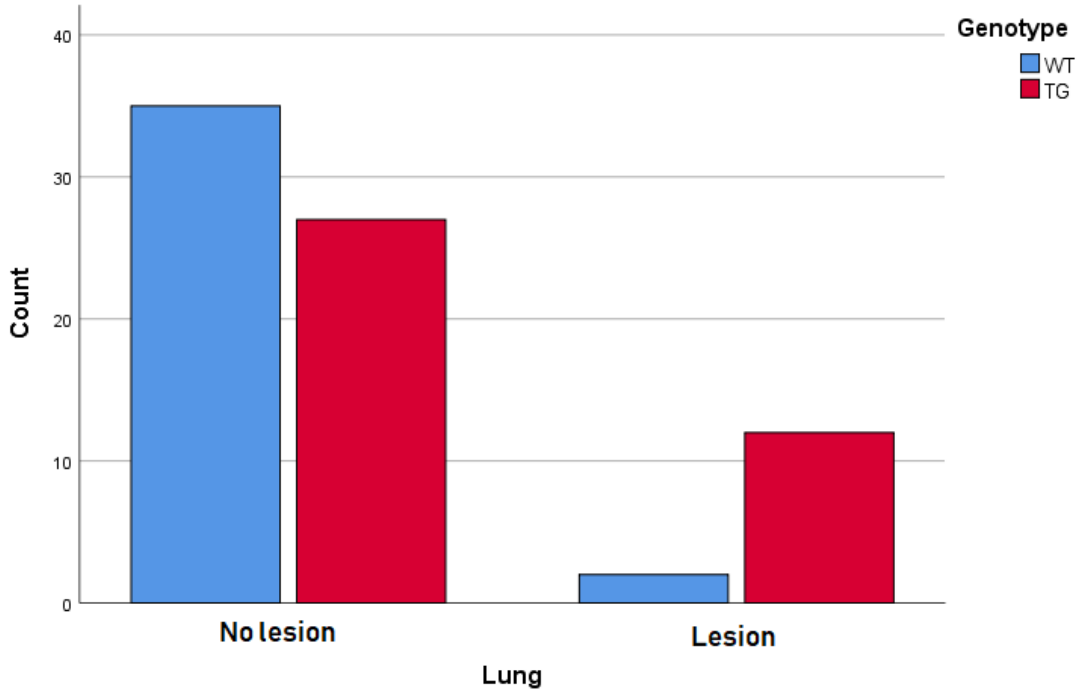


Fig. 12) Mineralization of the alveolar septa, lung. A - The alveolar septa show linear mineral deposits. HE. **B**- The mineral deposits are stained in black, confirming to be calcium. von Kossa.

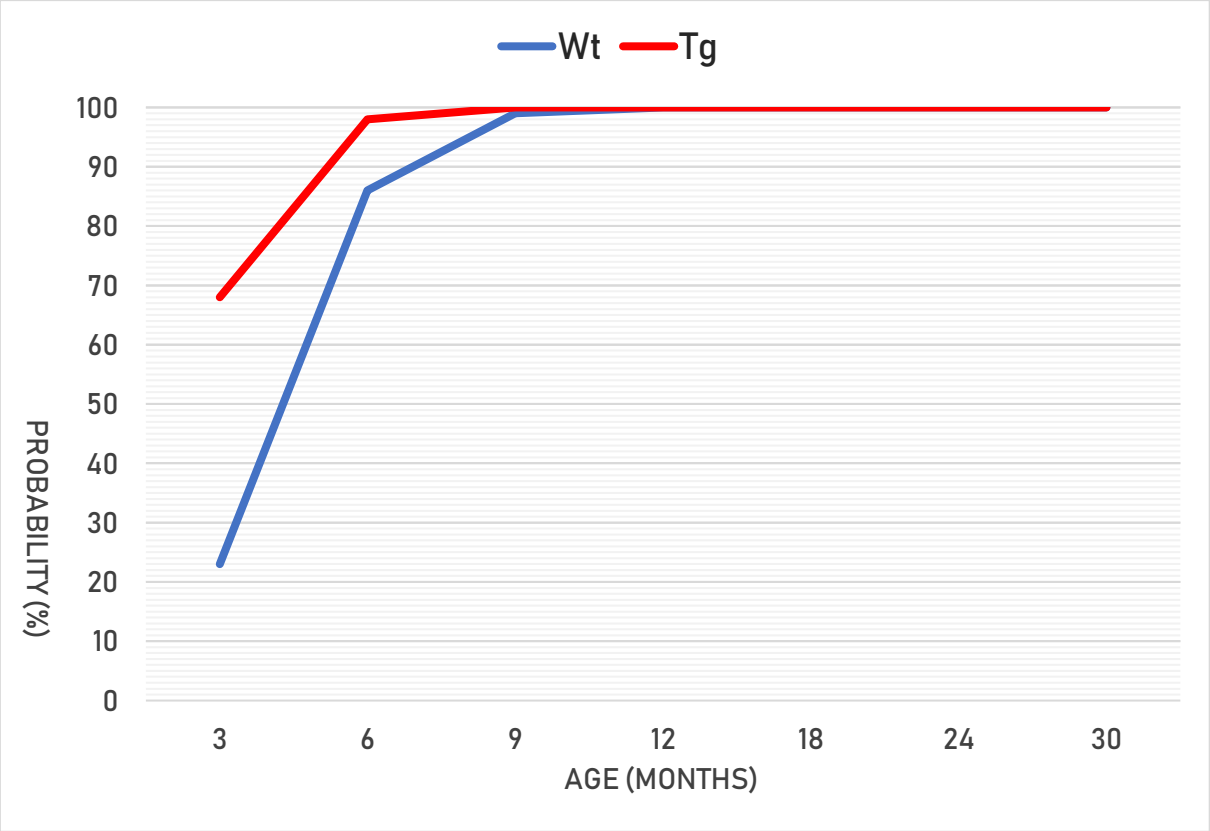
Alveolar histiocytosis was the most prevalent lesion both in Wt (one 77-week male and one 80-week-old female) and Tg (five females with mean age of 65 weeks and one 75-week-old male). Alveolar septa mineralization was only observed in Tg (three males and two females with mean ages of 103 and 89 weeks, respectively).

Tg rats are 7.7 times more likely to develop lung pathology than Wt rats [OR 7.7, IC 95% 1.604-37.19; ($p<0.05$)] (graphic 7).



Graphic 7) Lung pathology in Wt and Tg rats.

Logistic regression including on age and genotype revealed that both age and genotype had impact on lung pathology. For every additional week of age, the risk of onset of pathology is increased by 1.023 times [OR=1.025; ($p<0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg are 6.7 times more likely to develop lesions than Wt [OR=6.705; ($p<0.05$)]. A table with the calculation of the probability for occurrence of lung pathology in Wt and Tg rats per age group is presented below (Graphic 8).



Graphic 4) Probability of occurrence of lung pathology in Wt and Tg rats

3.1.3) Urinary system- Kidney

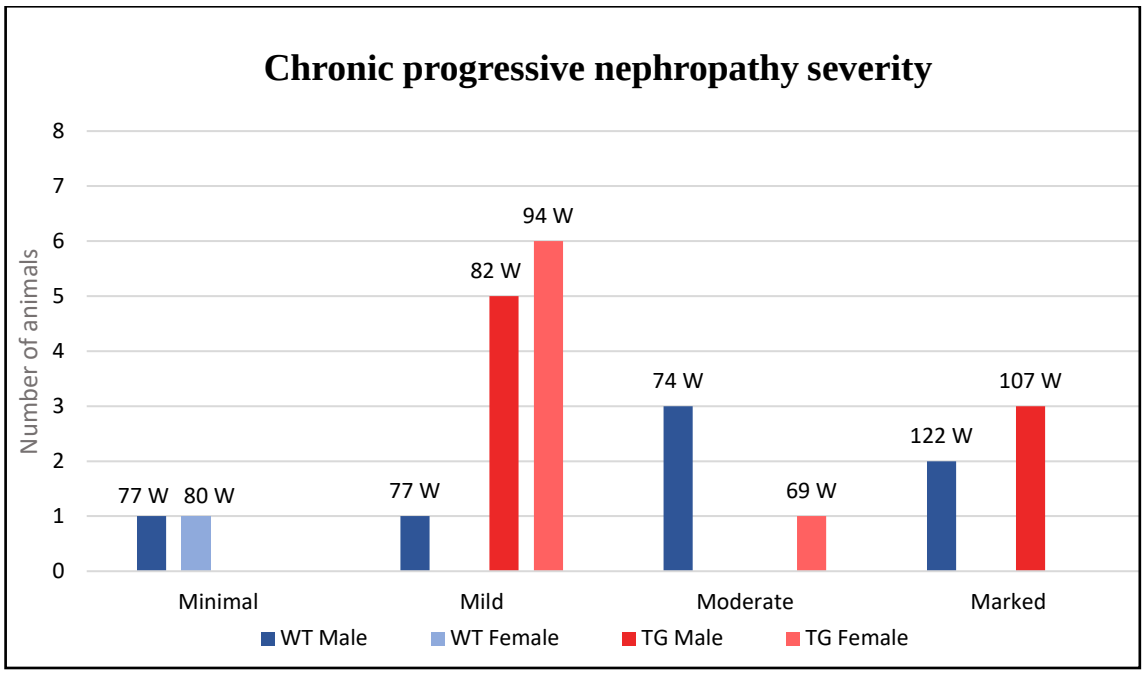
The spectrum, frequency and mean age of onset of kidney lesions are summarized in Table 5.

Diagnosis	Wt				Tg			
	Male		Female		Male		Female	
	n	**Age**	**n**	**Age**	**n**	**Age**	**n**	**Age**
Chronic progressive nephropathy	7	89	1	80	8	92	7	90
Mineralization, intraluminal	0	--	0	--	0	--	3	67
Pigment accumulation, tubular	0	--	3	67	2	49	4	71
Infarct	1	15	0	--	0	--	2	53

Table 5) Spectrum of kidney lesions in Wt and Tg rats. Age (weeks).

Overall, kidney lesions consisted on chronic progressive nephropathy, intraluminal mineralization, tubular pigment accumulation and infarct.

Chronic progressive nephropathy was the most frequent lesion. It consists of a combination of lesions such as areas or foci of tubular basophilia, with or without tubular hyperplasia; prominent hyaline casts within the lumen of the tubules; tubule atrophy and dilatation; glomerular sclerosis and atrophy; mononuclear inflammatory cell infiltration and interstitial fibrosis in advanced cases (Frazier et al., 2012). These can be focal, multifocal or regionally extensive, hence of different severity, for which they were classified using a score of 1 to 4 (minimal, mild, moderate, marked) (graphic 9) (fig. 13 and 14).



Graphic 9) Chronic progressive nephropathy score system in Wt and Tg (CaMKII-hA2AR) rats (W= weeks)

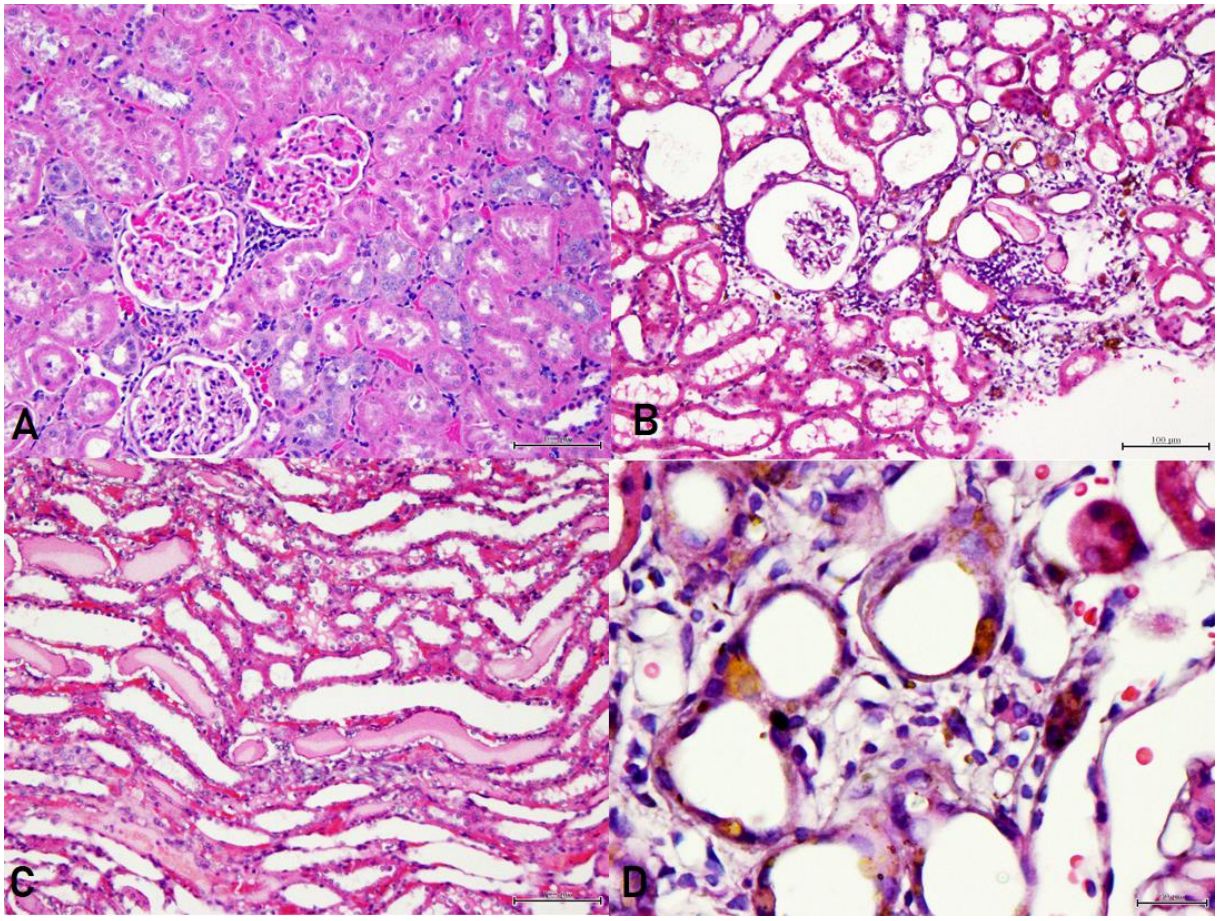


Fig.13) Chronic progressive nephropathy, kidney. **A)** Mild lesion with mononuclear inflammatory cell infiltration and tubular basophilia**. B, C)** Moderate lesion with tubular distension and atrophy, tubular protein casts mononuclear inflammatory cell infiltration, tubular pigment accumulation and mild fibrosis**. D)** Moderate lesion with tubular distension, atrophy and pigment accumulation

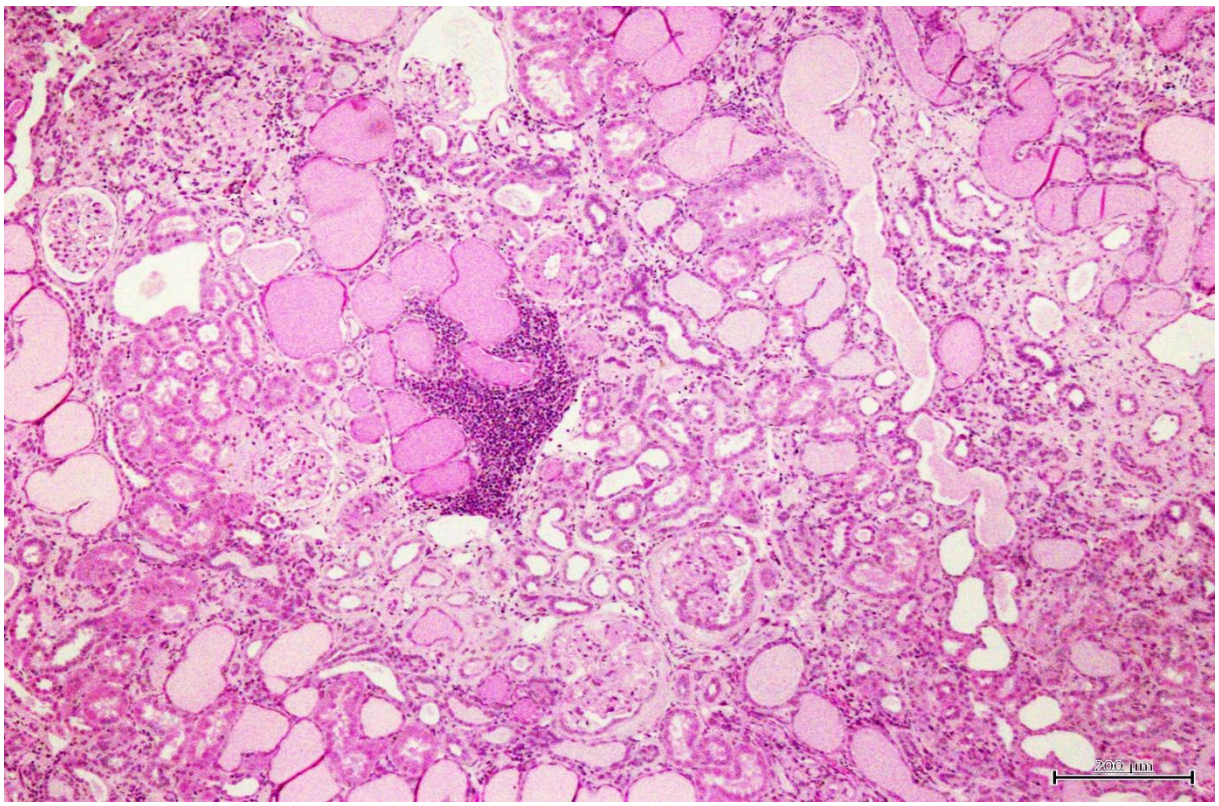
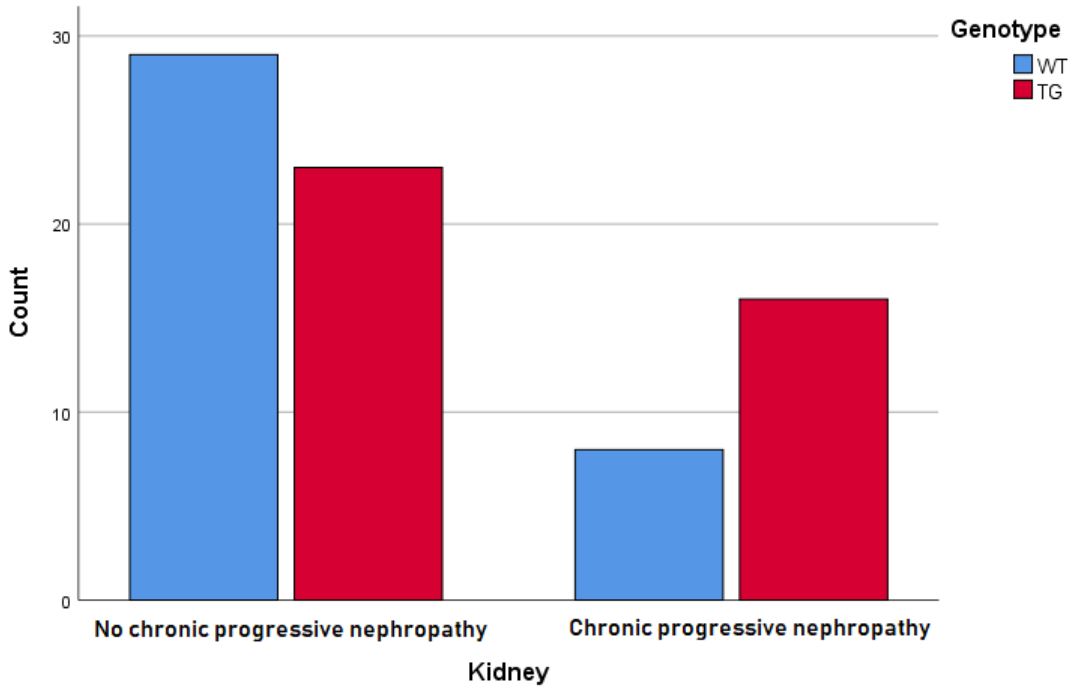


Fig.14) Chronic progressive nephropathy, marked, kidney. Marked lesion with multifocal areas with tubular protein casts, tubular distension and atrophy, interstitial fibrosis and mononuclear inflammatory cell infiltration.

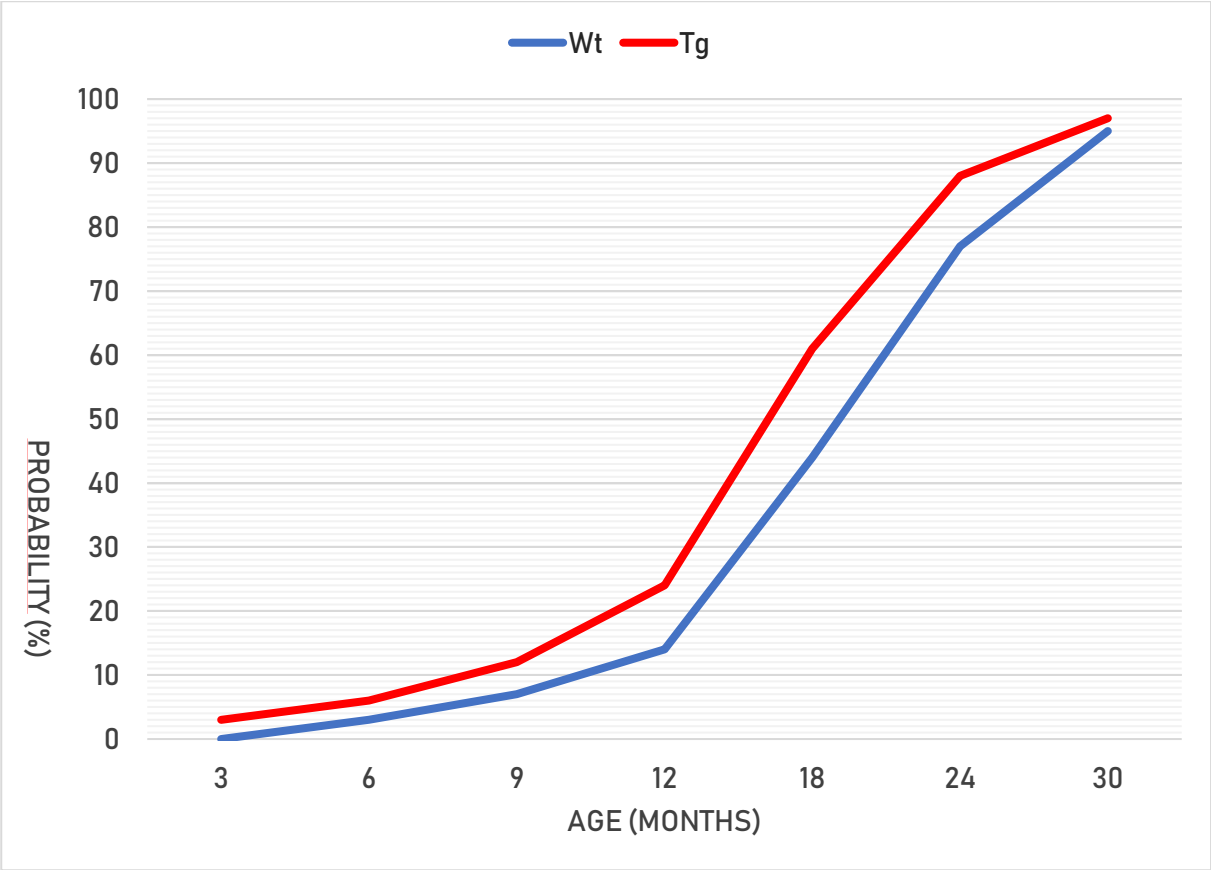
Chronic progressive nephropathy was the most prevalent kidney lesion in this study, both in Wt (seven males with mean age of 89 weeks and one 80-week-old female) and Tg rats (eight males and seven females with mean age of 92 and 90 weeks, respectively).

Tg rats are 2.5 times more likely to develop chronic progressive nephropathy than Wt rats. [OR 2.5, IC 95% 0.919-6.923; ($p>0.05$)]. In this case, however, as p value is greater than 0.05 the null-hypothesis is supported, for which these results cannot be extrapolated for the entire rat population (graphic 10).



Graphic 10) Chronic progressive nephropathy in Wt and Tg rats.

Logistic regression including age and genotype revealed that age was the only significant risk factor for the development of chronic progressive nephropathy and for every additional week of age the risk of onset of pathology is increased by 1.063 times [OR=1.063, ($p<0.05$)]. No significant difference was seen between Tg and Wt [OR 1.902, ($p>0.05$)]. A table with the calculation of the probability for occurrence of chronic progressive nephropathy in Wt and Tg rats per age group is presented below (Graphic 11).



Graphic 11) Probability of occurrence of chronic progressive nephropathy in Wt and Tg rats.

Renal chronic infarct was observed in Wt (one 15-week-old male) and Tg rats (three females with mean age of 53 weeks), consisting on variable scarring and interstitial fibrosis, with marked tubular atrophy, and tubular collapse with or without inflammation or dystrophic mineralization (Frazier et al., 2012).

Intraluminal mineralization of the tubules was only observed in Tg rats (three females, with mean age of 67 weeks). This lesion is characterized by the deposition of basophilic granular material or large amorphous to crystalline aggregates in the lumen of the tubules, most commonly found along the corticomedullary junction, but also in cortex, medulla, or papilla (Frazier et al., 2012) (fig. 15).

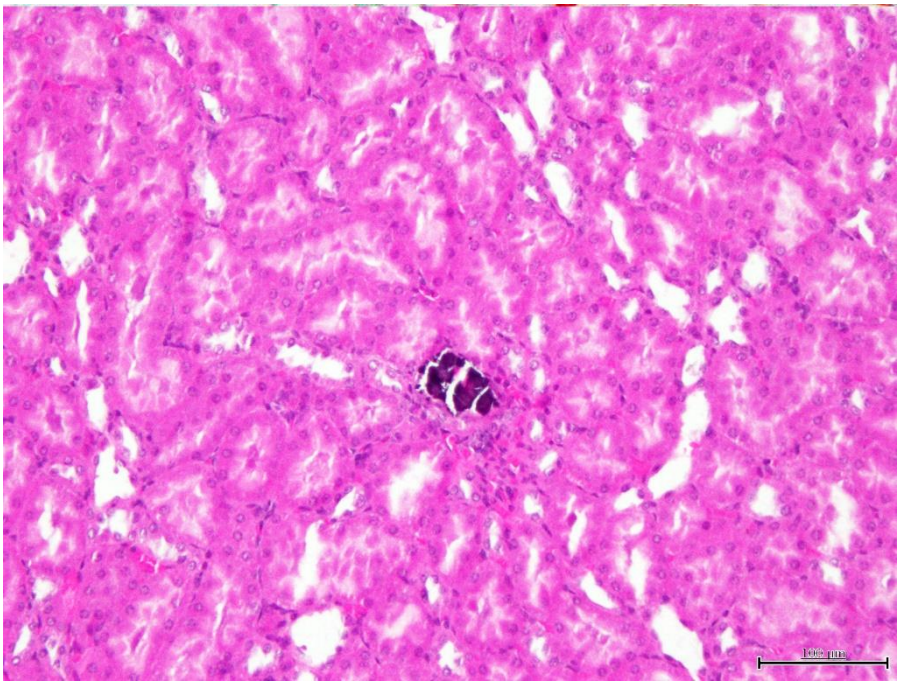


Fig. 15) Intraluminal mineralization, kidney. Deposition of basophilic granular material in the lumen of a tubule. HE.

Tubular pigment accumulation was observed in both Wt (three females with mean age of 67 weeks) and Tg rats (four cases in females and two in males, with mean age of 71 and 49 weeks, respectively). In order the understand the nature of the pigment, Prussian Blue staining was performed, and blue-stained granules were identified as hemosiderin whereas brown-stained granules were diagnosed as lipofuscin accumulation within the tubules (fig.16).

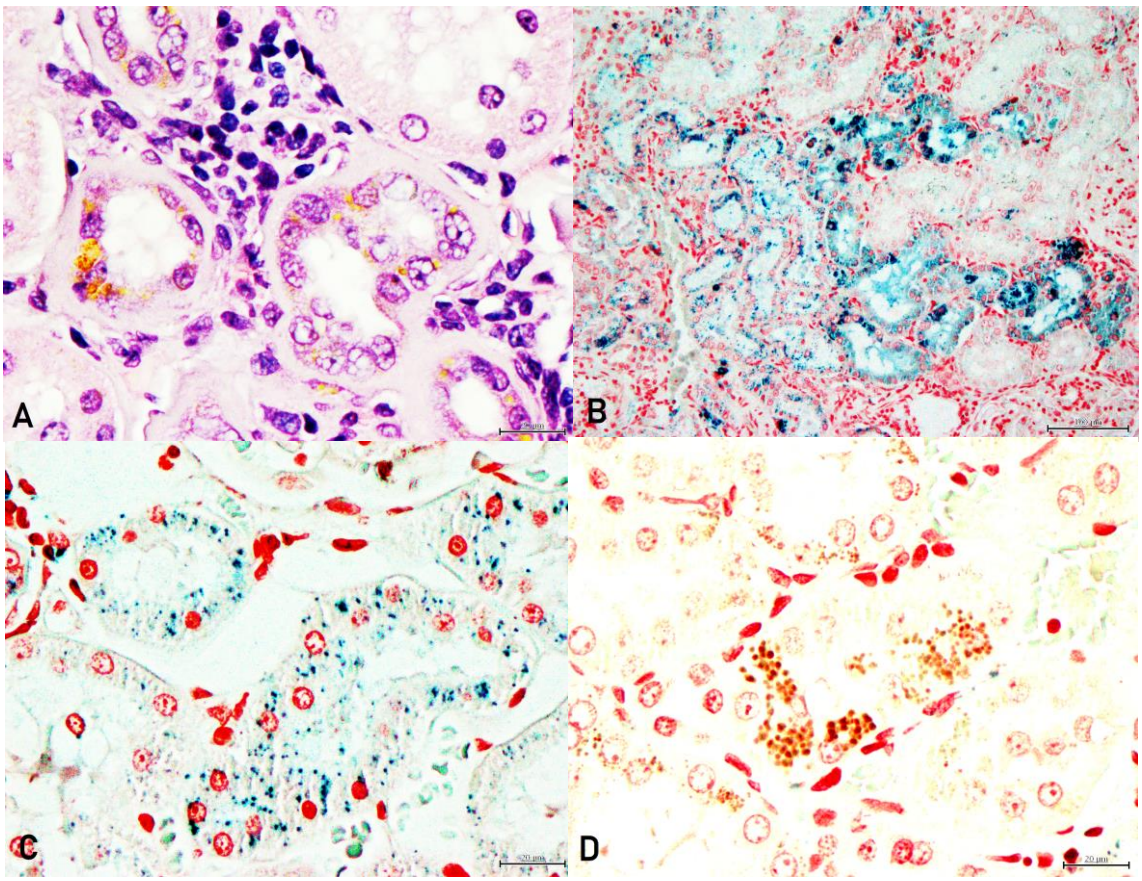


Fig.16) Tubular pigment accumulation, kidney. A) Accumulation of yellow or brow pigment in the cytoplasm of the epithelial tubular cells. HE**. B, C)** Cytoplasmic hemosiderin accumulation (blue pigment). Prussian Blue. **D)** Lipofuscin pigment accumulation (brown pigment), negative for Prussian Blue. Prussian Blue.

3.1.4) Hepatobiliary system- Liver

The spectrum, frequency and mean age of onset of hepatic lesions are summarized in Table 6.

Diagnosis	Wt				Tg			
	Male		Female		Male		Female	
	n	Age	n	Age	n	Age	n	Age
Lipidosis	3	56	1	37	0	--	1	69
Fibrosis, periportal	2	77	2	83	2	22	2	48
Hyperplasia, bile duct	2	102	1	88	1	32	3	87
Extramedullary hematopoiesis	2	48	2	45	3	72	1	66
Mononuclear inflammatory cell infiltration	2	39	3	58	6	72	13	82

Table 6) Spectrum of hepatic lesions in Wt and Tg rats. Age (weeks).

Overall, liver lesions included lipidosis, periportal fibrosis, bile duct hyperplasia, extramedullary hematopoiesis and mononuclear inflammatory cell infiltration.

Lipidosis was observed in Wt (three males with mean age of 56 weeks and one 37-week-old female) and Tg rats (one 69-week-old female). This lesion consists of multifocal areas of macro and/or micro vesicular changes in the hepatocytes, which acquire a foamy aspect (Thoolen et al., 2010).

Periportal fibrosis was observed in both Wt (two males and two females, with mean age of 77 and 83 weeks) and Tg rats (two males and two females, with mean age of 22 and 42 weeks). This lesion consists of the presence of connective tissue (fibrosis) in the periportal areas of the liver (fig.17A).

Bile duct hyperplasia was seen both in Wt (two males with mean age of 102 weeks and one 88-week-old female) and Tg rats (one 32-week-old male and three females with mean age of 102 weeks). This lesion consists of an increased number of small bile ducts arising in portal region; the biliary epithelium is well differentiated, forming normal ducts and may show degenerative changes (Thoolen et al., 2010) (fig. 17B).

Extramedullary hematopoiesis was seen both in Wt (two males and two females with mean age of 48 and 45 weeks, respectively) and Tg rats (three males with mean age of 72 weeks and one 66-week female). This lesion consists of multifocal to coalescent foci of numerous hematopoietic cells, including immature myeloid and erythroid cells, as well as occasional megakaryocytes, in the liver sinusoids (Thoolen et al., 2010) (fig. 17C).

Mononuclear inflammatory cell infiltration was seen both in Wt (two males and three females, with mean ages of 39 and 58 weeks, respectively) and Tg rats (six males and thirteen females, with mean ages of 72 and 82 weeks). This lesion consists of the multifocal to coalescent infiltration of plasma cells, lymphocytes and rare macrophages within the liver parenchyma (Thoolen et al., 2010) (fig.17D).

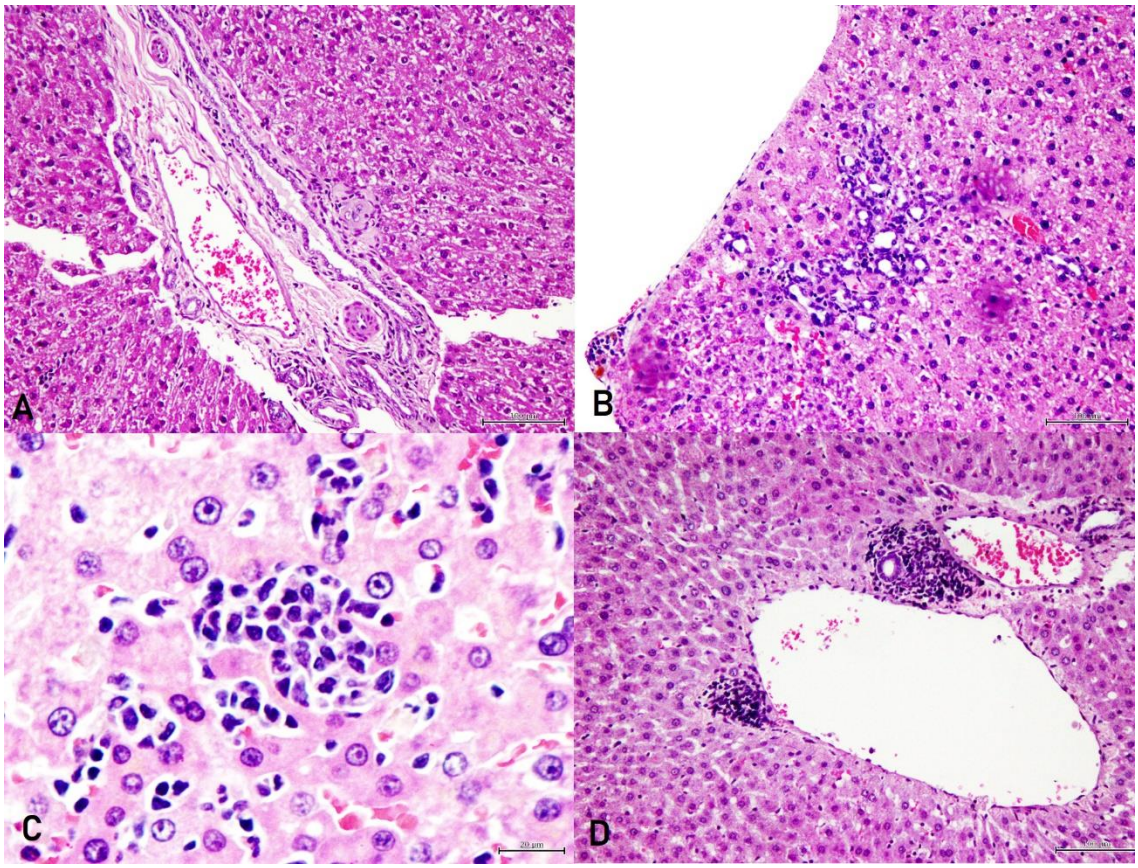


Fig. 17) Liver. A) Periportal fibrosis, focal, minimal. **B)** Bile duct hyperplasia, focal, mild**. C)** Extramedullary hematopoiesis. **D)** Mononuclear inflammatory cell infiltration, multifocal, mild. HE

3.1.5) Endocrine system

3.1.5.1) Adrenal gland

The spectrum, frequency and mean age of onset of adrenal lesions are summarized in Table 7. Only 42 adrenals were analyzed (24 Wt: 17 males and 7 females; 18 Tg: 11 males and 7 females).

	Wt				Tg			
Diagnosis	Male		Female		Male		Female	
	n	**Age**	**n**	**Age**	**n**	**Age**	**n**	**Age**
Vacuolation, cortical, cytoplasmatic	7	77	2	64	9	50	4	67
Adrenocortical accessory nodule	2	59	0	--	2	38	2	41
Lipogenic pigment, cortical, cytoplasmatic	1	40	3	68	2	115	2	53

Table 7) Spectrum of adrenal lesion in Wt and Tg rats. Age (weeks).

Overall, adrenal lesions consisted of vacuolation of the cortical cells, adrenocortical accessory nodule and lipogenic pigment accumulation in cortical cells.

Vacuolation of the cortical cells was the most frequent lesion. This lesion is characterized by the accumulation of clear vacuoles within cortical cells, mainly in the zona fasciculata, although the zonae reticularis and glomerulosa are also often affected; and can be focal or diffuse (Hoenerhoff, Gruebbel, & Gill, 2014) (fig.18).

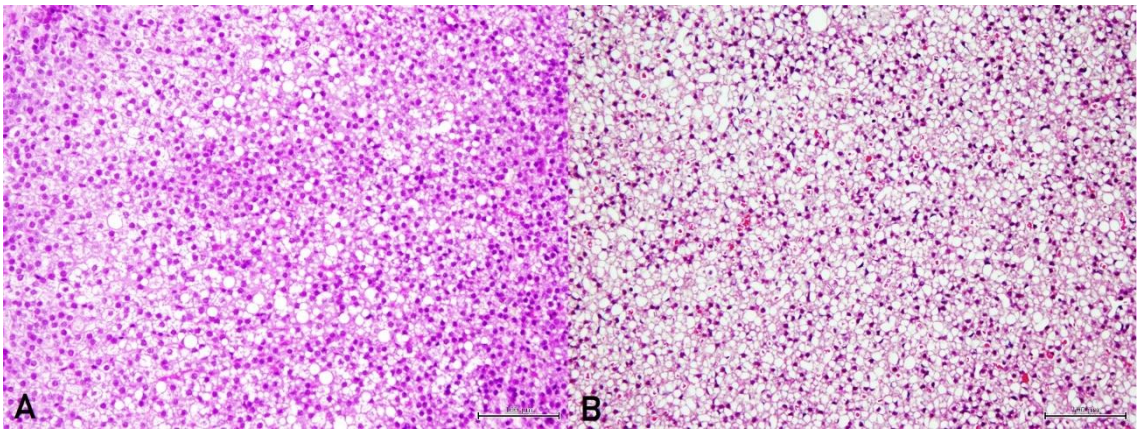
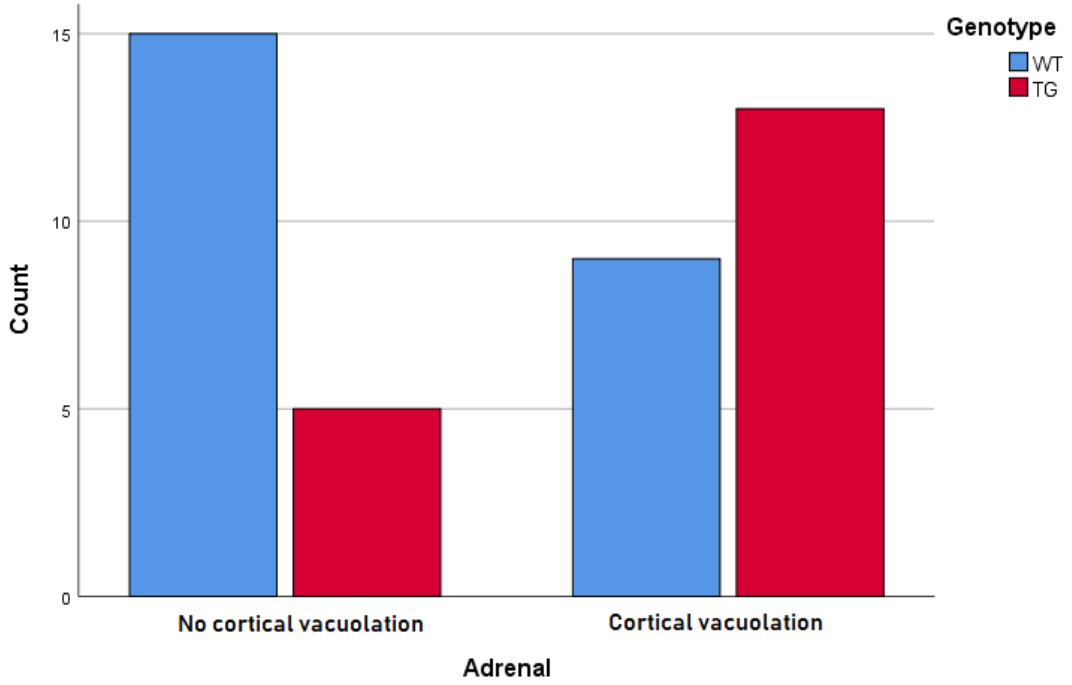


Fig.18) Vacuolation, cortical, adrenal gland. A) Moderate, multifocal. **B)** Marked, diffuse. HE.

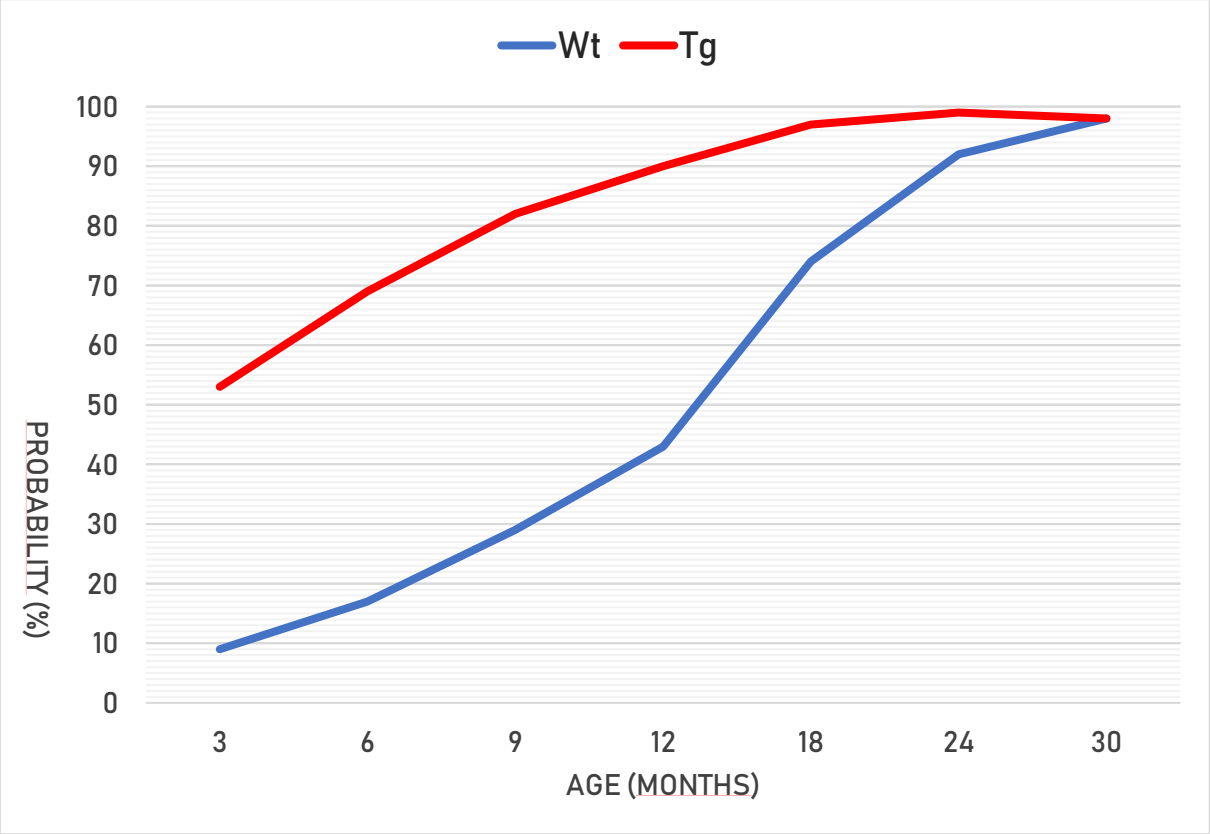
Vacuolation of the cortical cells was the most frequent lesion both in Wt (seven males and two females, with mean age of 77 and 64 weeks, respectively) and Tg rats (nine males and four

females, with mean ages of 50 and 67 weeks, respectively). Tg rats are 4.3 times more likely to develop pathology than Wt rats [OR 4.3, IC 95% 1.156-16.248; ($p<0.05$)] (graphic 12).



Graphic 12) Cortical vacuolation in Wt and Tg rats.

Logistic regression including age and genotype revealed that both age and genotype had an impact on cortical vacuolation. For every additional week of age, the risk of onset of pathology is increased by 1.005 times [OR=1.005; ($p<0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg are 8.5 times more likely to develop this lesion than Wt [OR=8.548; ($p<0.05$)]. A table with the calculation of the probability for occurrence of cortical vacuolation in Wt and Tg rats per age group is presented below (Graphic 13).



Graphic 5) Probability of occurrence of vacuolation of the cortical cells in Wt and T rats

Adrenocortical accessory nodule was found in both Wt (two males, with mean age of 59 weeks) and Tg rats (two males and two females, with mean age of 38 and 41 weeks, respectively). This lesion is characterized by the presence of concomitant adrenocortical tissue outside or immediately inside the adrenal capsule, most commonly found in the retroperitoneal fat adjacent to the adrenal gland or kidney but can be found anywhere in the abdominal cavity. These nodules are composed of normal cortex either detached from the adrenal gland or attached to the gland and may be separated by a fibrous capsule, without compression of the adjacent parenchyma (Brändli-Baiocco et al., 2018) (fig.19A)

Lipogenic pigment was found in the cytoplasm of the cortical cells of the adrenal gland in both Wt (one 40-week-old male and three cases in females with mean age of 68 weeks) and Tg rats (two males and two females, with mean age of 40 and 68 weeks, respectively). Histologically, this lesion is characterized by the presence of yellow to brown granular pigment, usually in cells of the zona reticularis and/or histiocytes at the junction of the zona reticularis and medulla (Brändli-Baiocco et al., 2018) (fig19B)

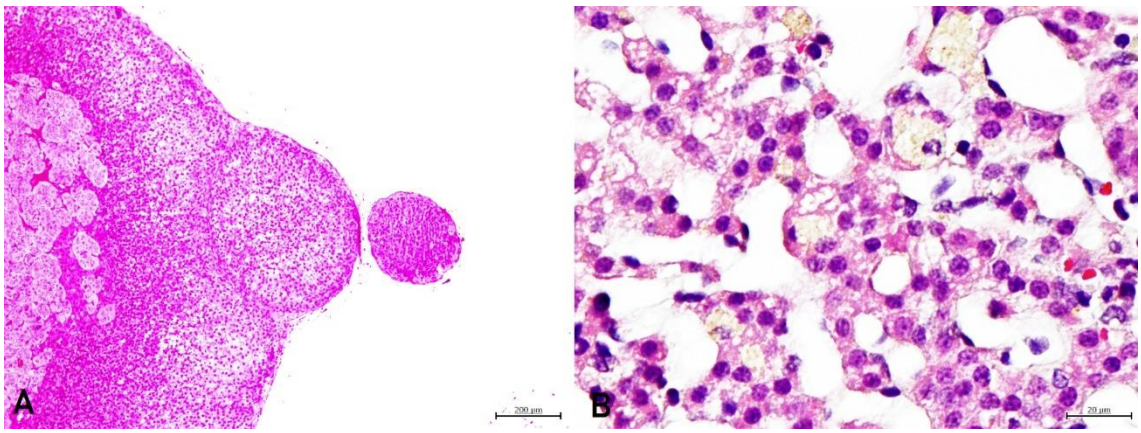


Fig 19) Adrenal gland. A) Adrenocortical accessory nodule. **B)** Lipogenic pigment in the cytoplasm of cortical cells. HE.

3.1.5.2) Thyroid

Thyroid follicular hypertrophy was diagnosed in three females, two of them Wt rats with mean age of 64 weeks and one Tg rat with 118 weeks. This lesion consists of thyroid follicles lined by cuboidal to columnar epithelium with central follicles tightly packed and smaller than normal (fig 20).

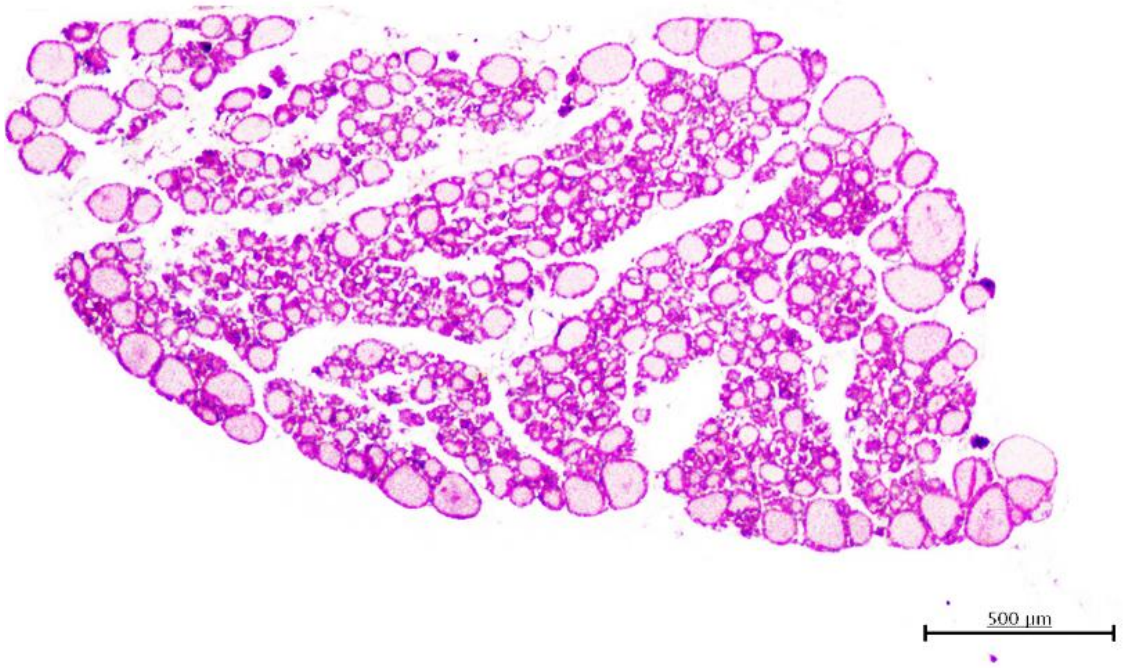


Fig.20) Thyroid follicular hypertrophy, thyroid. Thyroid follicles lined by cuboidal to columnar epithelium with central follicles tightly packed and smaller than normal. HE

3.2) Proliferative lesions

3.2.1) Mammary fibroadenomas

Mammary fibroadenomas were diagnosed both in Wt (one 77-week-old female) and Tg rats (five females with mean age of 79 weeks). Clinical examination of these animals revealed large, firm, circumscribed masses extending from the cervical region to the inguinal area. Grossly, these tumors are solid, well demarcated masses, smooth-surfaced, firm, pale-tan to tan. Microscopically, they are well defined and uniform structures with epithelial and connective tissue components, classified as mammary fibroadenomas. Ducts and acini are surrounded by layers of proliferative fibrous tissue, usually with low mitotic index (Rudmann et al., 2012) (fig.21).

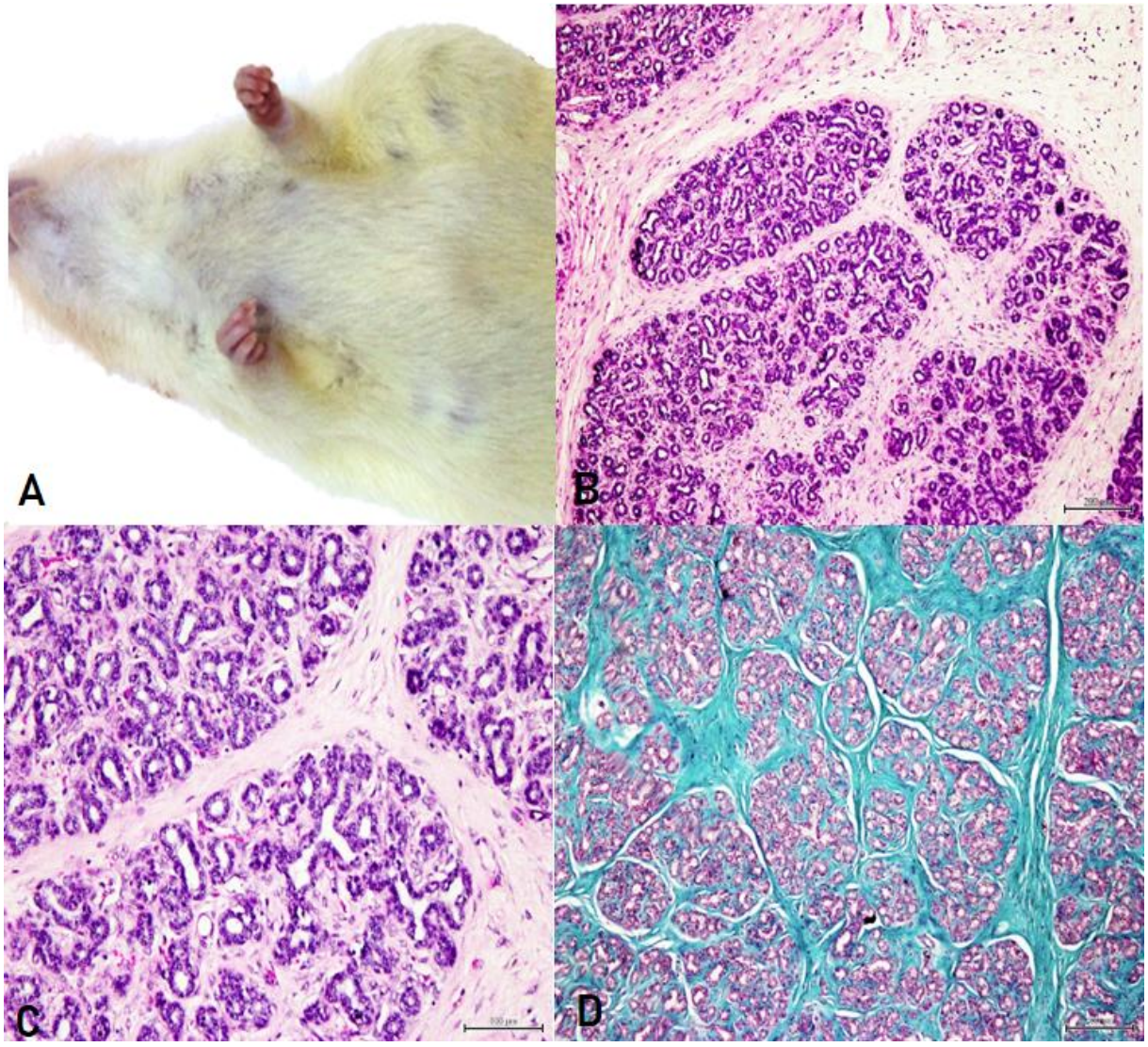


Fig. 21) Mammary fibroadenoma, mammary gland. A) Macroscopic aspect of the mammary fibroadenoma. **B, C,)** Microscopic aspect of the mammary fibroadenoma. HE. **D)** The fibrous tissue is evidenced with the blue staining. Masson's Trichrome.

3.2.3) Other proliferative lesions

A single case of multicentric lymphoma was also observed in a 75-week old Wt rat. Macroscopically, hepatomegaly and splenomegaly were observed and at the cut section, the parenchyma was infiltrated by multifocal pinpoint to one-millimeter foci, coalescent, tan to white and moderately firm. Microscopically, dense sheets of round neoplastic lymphoid cells extensively infiltrated and partially effaced the liver and spleen parenchyma, with disruption of tissue architecture. Cellular pleomorphism was low and a moderate mitotic index was observed (6 to 10 mitosis/400x filed) (fig.22)

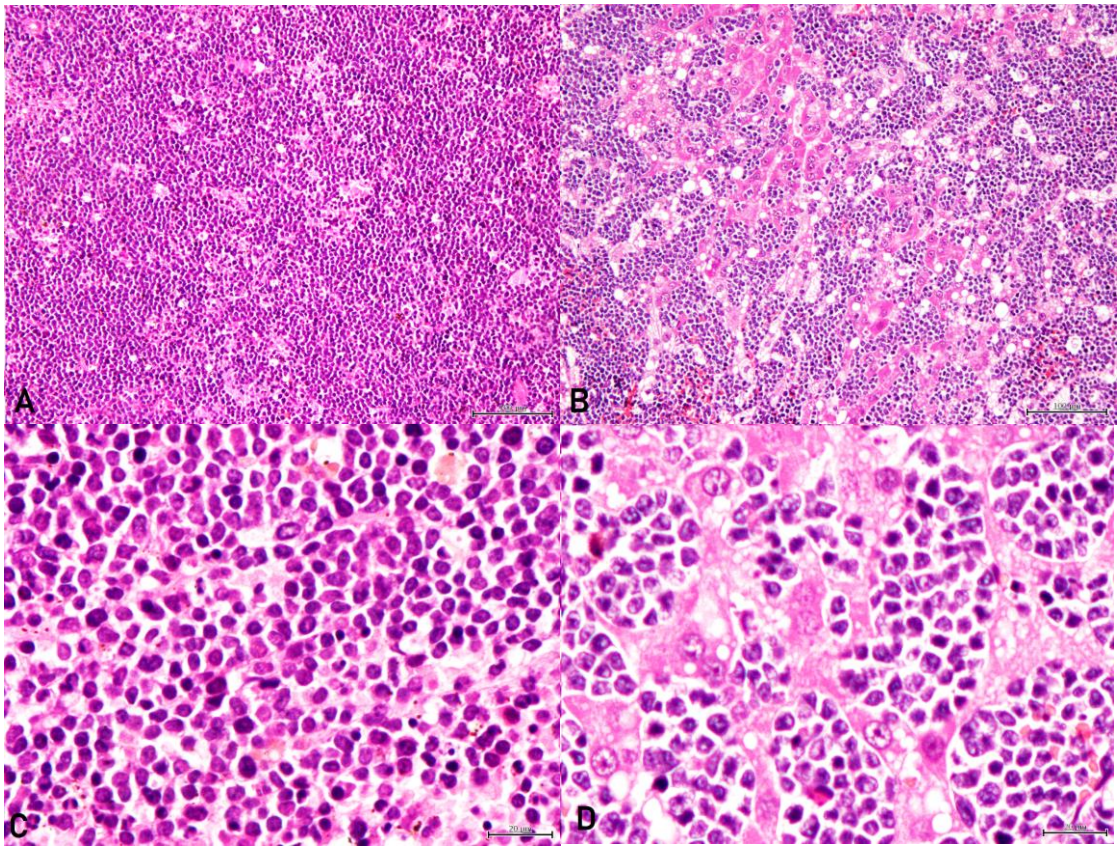


Fig. 22) Lymphoma, liver and spleen. A) Low magnification of lymphoma in the spleen**. B)** Low magnification of lymphoma in the liver. **C)** High magnification of lymphoma in the spleen. **D)** High magnification of lymphoma in the liver. HE

4. Discussion

In the present dissertation, we performed a longitudinal aging study in Sprague- Dawley rats. Our samples consisted on males (n=42) and females (n=34) of two different genetic backgrounds, Wt (n=37) and Tg (Tg (CaMKII-hA2AR)) (n=39). We therefore describe the spectrum of spontaneous age-associated lesions that occur in these animals, and also perform statistical analysis using SPSS to evaluate the impact of the genotype of these rats in aging.

Overall, Tg rats were 2.7 times more likely to develop pathology than Wt rats [OR 2.745, IC 95% 1.0.07-6.997 ($p<0.05$)]. When controlled the statistical analysis for age (i.e. age matched animals) Tg rats were 2.8 times more likely to develop age-associated lesions than Wt [OR=2.8, ($p>0.05$)]. We also found that age was the most determinant factor for the occurrence of lesions and for every additional week of age the risk of onset of pathology increased by 1.060 times (OR 1.060, $p<0.05$). Since aging is defined as a time-dependent functional decline, progressive loss of physiological integrity and progressive increase in disease susceptibly (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Franceschi & Campisi, 2014; Mitchell, Scheibye-Knudsen, Longo, & de Cabo, 2015), it was expected to have an increase of risk of onset of pathology for every extra week of age. Similar analysis was performed for cardiovascular, endocrine, pulmonary and renal pathology.

Regarding the heart, cardiomyopathy was the most prevalent cardiac lesion in this study, both in Wt (eight males and four females with mean age of 78 and 83 weeks, respectively) and Tg rats (five males and six females with mean age of 84 and 85 weeks, respectively), and its incidence did not differ between groups [OR 0.82; IC 95% 0.315-2.139, ($p>0.05$)].

Progressive cardiomyopathy is a relatively common background lesion in experimental and toxicological studies and this term is used to describe a spectrum of morphologic changes that include cardiomyocyte degeneration, necrosis, inflammation and fibrosis (Berridge et al., 2016). It affects different strains of laboratory rats and mice and its incidence increases with age (Chanut et al., 2013). According to our results, age was in fact the most significant risk factor for the development of cardiomyopathy and for every additional week of age the risk of onset of pathology is increased by 1.046 times [(OR=1.046; ($p<0.05$)], and males were usually more affected than females. However, this lesion can develop in rats as young as 12 weeks of life (Rothenburger, Himsworth, Treuting, & Leighton, 2015; Hailey et al., 201).

The etiology of this disorder is still unknown, although recent studies suggest that this change could be a consequence of local vascular dysfunction as the most affected areas of the heart are subject to higher blood flow and oxygen requirement, and thus are more at risk for ischemia (Chanut et al., 2013). Nutrition is also believed to have a role on the pathogenesis of the disease

as Sprague-Dawley and Wister rats on restricted caloric diet have lower incidence of cardiomyopathy (Chanut et al., 2013).

Vacuolar degeneration was the second most common finding in the hearts and it was observed both in Wt (one male with 28-weeks and four females with mean age of 38 weeks) and Tg rats (one 32-week-old male and four females with mean age of 38 weeks). It is reported that these vacuoles in cardiomyocytes are often correspond to an abnormal accumulation of energy subtracts such as lipids, to altered processing of phospholipid membranes or to dilatation of specific organelles such as mitochondria or sarcoplasmic reticulum (Berridge et al., 2016). The differential diagnosis cannot be done by light microscopy and other techniques such as electron microscopy (Berridge et al., 2016) are necessary to unravel the nature of the vacuoles. Herein, univariate and multivariate analysis was only performed for cardiomyopathy cases. Vacuolar degeneration was not subject to univariate and multivariate analysis since it represented a rare event, as opposed to cardiomyopathy.

Regarding blood vessels, mineralization was the most frequent vascular lesion, both in Wt (two males and two females, with mean age of 56 and 58 weeks, respectively) and Tg rats (nine males and nine females, with mean age of 62 and 87 weeks, respectively). Tg rats were found to be 5.5 times more likely to develop vascular pathology than Wt rats [OR 5.486, IC 95% 1.776- 17.074; ($p<0.05$)]. Logistic regression including age and genotype revealed that age had a low impact in the development of vascular mineralization [OR 1.013; ($p>0.05$)]. When we control for age and compare Wt and Tg rats (i.e. age-matched animals), Tg's were 4.9 times more likely to develop lesions than Wt [OR=4.954; ($p<0.05$)].

Vascular mineralization is usually seen in aged Sprague-Dawley rats as a consequence of chronic progressive nephropathy, associated with secondary hyperparathyroidism (Berridge et al., 2016). However, in our study, no correlation was found between vascular calcification and renal disease. Other causes for vascular calcifications also include metabolic syndrome and endocrine pathology that lead to deregulation of calcium metabolism and homeostasis. Both in humans and animals, a chronic pro-inflammatory status is a common feature of aging and is characterized by a chronic, low grade and generalized inflammatory status, also known as "inflammaging", in the absence of an infectious process. We reason that this process can also lead to vascular pathology. For instance, monocytes and macrophages accumulated in the artery wall can enhance calcification through the release of TNF-a. Also basic calcium phosphate internalized by the macrophages further increase their cytokine production and consequent promotion of calcifications (Shantsila & Lip, 2009).

Regarding the lung, alveolar histiocytosis was the most prevalent lesion both in Wt (one 77-week male and one 80-week-old female) and Tg rats (five females with mean age of 65 weeks

and one 75-week-old male). Alveolar septa mineralization was only observed in Tg rats (three males and two females with mean ages of 103 and 89 weeks, respectively). These were 7.7 times more likely to develop lung pathology than Wt rats [OR 7.7, IC 95% 1.604-37.19; ($p<0.05$)].

Logistic regression analysis including age and genotype revealed that both factors had an impact on lung pathology. For every additional week of age, the risk of onset of pathology was increased by 1.023 times [OR=1.025; ($p<0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg's were 6.7 times more likely to develop lesions than Wt [OR=6.705; ($p<0.05$)].

Alveolar histiocytosis consists of the accumulation of foamy macrophages in the alveoli and can be induced in rats by alteration of endogenous lipid metabolic processes; by hypophysectomy; in pulmonary necrosis being one of the primary lung responses to inhaled toxicants (Renne et al., 2009). Aging can also contribute to the pathogenesis of this processes since multifocal alveolar histiocytosis is also seen in aged rats (Renne et al., 2009). Since none of the animals in our study had contact to toxicants and no necrotic processes were diagnosed in the lung, we assume that alveolar histiocytosis was most likely age-related. Alveolar histiocytosis is considered an inflammatory process, characterized by the invasion of alveolar lumen by macrophages. These changes could be due the aging process and "inflammaging" discussed in the introduction. This inflammatory microenvironment leads to progressive tissue degeneration and represents a significant risk factor for morbidity and mortality in the elderly (Sanada et al., 2018).

Mineralization of the alveolar septa is usually observed in severe cases of chronic progressive nephropathy in aged rats and may be accompanied by increased macrophage aggregates or acute serous inflammation (Renne et al., 2009). Chronic progressive nephropathy was only observed in three cases, which can explain the alveolar septa mineralization. The other two cases had no changes in the kidney, therefore the etiology was considered non-related to chronic progressive nephropathy.

Regarding the kidney, chronic progressive nephropathy was the most prevalent kidney lesion in this study, both in Wt (seven males with mean age of 89 weeks and one 80-week-old female) and Tg rats (eight males and seven females with mean age of 92 and 90 weeks, respectively).

Tg rats were 2.5 times more likely to develop chronic progressive nephropathy than Wt rats. [OR 2.5, IC 95% 0.919-6.923; ($p>0.05$)]. Logistic regression including age and genotype revealed that age was the only significant risk factor for the development of chronic progressive nephropathy and for every additional week of age the risk of onset of pathology is increased by

1.063 times [OR=1.063, ($p<0.05$)]. No significant difference was seen between age-matched Tg and Wt [OR 1.902, ($p>0.05$)].

Chronic progressive nephropathy, also known as chronic kidney disease, is a very common age-related disorder observed in several strains of rats used in research such as Sprague- Dawley and Fischer 344 rats (Hard & Khan, 2004). It occurs in both sexes but, like in our study, males have higher incidence than females (Frazier et al., 2012). High-protein diets are an important contributing factor and high prolactin levels have been implicated as a contributing factor. However, the clear etiology of chronic progressive nephropathy is still unknown (Frazier et al., 2012; Hard & Khan, 2004). As aging advances, it can progress to an end-stage kidney disease leading to chronic renal failure and eventually death. Besides, since chronic progressive nephropathy is both regenerative and degenerative, the high rate of cellular turnover and proliferation increases the risk for renal tumor development (Hard et al., 2013).

Intraluminal mineralization was diagnosed in three Tg female rats, with mean age of 67 weeks. This lesion consists of mineral deposits within the tubules as a sequel of tubular degeneration or necrosis and can be an early secondary consequence of renal failure. In chronic progressive nephropathy, a deficit of vitamin D and abnormal calcium sensor receptors are important factors in the beginning of the process. As the disease progresses, hyperphosphatemia becomes and additional risk factor (Frazier et al., 2012). Like in our study, this lesion is more frequent in females rather than males (Frazier et al., 2012).

Tubular pigment accumulation was observed in both Wt (three males with mean age of 67 weeks) and Tg rats (two males and four females with mean age of 40 and 71 weeks, respectively). Both lipofuscin and hemosiderin were observed within the renal tubules. Lipofuscin accumulation is considerate an age-related disorder and consists of yellow to brownish pigment granules composed by residues of lysosomal digestion (Frazier et al., 2012). On the other hand, hemosiderin accumulation in the tubules usually results from hemoglobinuria nephropathy, which can result in severe kidney failure (Frazier et al., 2012).

Univariate and multivariate analysis was only performed for chronic progressive nephropathy cases. Intraluminal mineralization and tubular pigment accumulation were not included in the analysis since these lesions were classified has minimal and mild and were considered of reduced importance, as opposed to chronic progressive nephropathy.

Regarding the liver, lipidosis was diagnosed in both Wt (three males with mean age of 56 weeks and one 37-week-old female) and Tg rats (one 69-week-old female). Hepatic lipidosis is a frequent lesion in rats and it can be induced by several agents including xenobiotics but can also be observed spontaneously, which is what most likely happened within our population of rats.

We also observed periportal fibrosis in both Wt rats (two males and two females with mean age of 77 and 83 weeks, respectively) and Tg rats (two males and two females with mean age of 22 and 48 weeks, respectively). Fibrosis is a reaction of the organism to prolonged damage and it is moderately common in aged rats being econsidered an inflammatory, proliferative and metaplastic reaction involving the liver (Thoolen et al., 2010) As aging advances, a persistent proinflammatory status takes place and there's a marked increase in cytokines such as TGF-B, a profibrogenic cytokine produced by inflammatory cells, Kupffer cells and hepatic stellate cells that has several effects on inflammation, extracellular matrix turnover and hepatocellular proliferation (Furtado et al., 2014). Therefore, fibrosis observed in our population could be due to aging and this persistent inflammatory status. This lesion was seen both in males and females but with earlier incidence in males. This could be to hormonal changes, as estrogen has an antifibrotic role and reduces hepatic fibrosis in rats (Xu et al., 2002).

Bile duct hyperplasia was seen in both Wt (two males with mean age of 102 weeks and one 88-week-old female) and Tg rats (one 32-week-old male and three females, with mean age of 87 weeks). Like fibrosis, this is also related to hepatic injury and it's considerate an spontaneous and age-related change more commonly seen in rats than in mice (Thoolen et al., 2010).

Extramedullary hematopoiesis was observed in both Wt (two males and two females with mean age of 48 and 45 weeks, respectively) and Tg rats (three males with mean age of 72 weeks and one 66-week-old female). It consists of small aggregates of hematopoietic cells randomly distributed in the hepatic sinusoids and it's observed in the liver in cases of hematopoietic demand and in situation of stress, anemia, infections, pregnancy and neoplasia (Thoolen et al., 2010).

Mononuclear inflammatory cell infiltration was the most commonly diagnosed lesion in both Wt (two males and three females with mean age of 39 and 58 weeks, respectively) and Tg rats (six males and thirteen females, with mean age of 72 and 82 weeks, respectively) This lesion is characterized by the infiltration of lymphocytes, plasma cells and macrophages in the hepatic parenchyma and it is due to a persistent noxious stimulus, such as the changes in the microenvironment during the aging process already discussed above.

Since all of the observed hepatic lesion were minimal to mild, no comparative studies between Wt and Tg rats were performed.

Regarding the adrenals, vacuolation of the cortical cells was the most frequent lesion both in Wt (seven males and two females, with mean age of 77 and 64 weeks, respectively) and Tg rats (nine males and four females, with mean ages of 50 and 67 weeks, respectively). Tg rats were 4.3 times more likely to develop this pathology than Wt rats [OR 4.3, IC 95% 1.156-16.248; ($p<0.05$)].

Logistic regression analysis including age and genotype revealed that both factors had impact on cortical vacuolation. For every additional week of age, the risk of onset of the pathology increased 1.005 times [OR=1.005; ($p<0.05$)]. When we control the statistical analysis for age and compare Wt and Tg rats (i.e. age-matched animals), Tg rats were 8.5 times more likely to develop the lesion than Wt [OR=8.548; ($p<0.05$)].

Cortical vacuolation can occur as a spontaneous and age-related change in rodents, especially in rats. It can also appear as a secondary change to chronic stress from variable causes; drugs and toxins, specialty the ones that interfere with the HPA axis; and can be a result of excess of ACTH or corticosterone (Hoenerhoff, Gruebbel, & Gill, 2014).

Adrenocortical accessory nodules were found in both in Wt (two males with mean age of 59 weeks) and Tgrats (two males and two in females, with mean age of 38 and 41 weeks, respectively). These nodules are fairly common both in rats and mice and are considerate developmental anomalies arising from the reminiscent or the coelomic epithelial primordia from which the adrenal cortex is derived. Although innocuous, they can develop degenerative, hyperplastic and neoplastic changes during the animal's lifetime. These nodules can be seen in young and older rats. However, a study of age-related changes in the adrenals revealed higher number of nodules in older rats (Parker & Valerio, 1996). Nevertheless, since the etiology of this lesion is embryogenic, the cases of adrenocortical accessory nodule were not included in the comparative study.

In summary, we found that Tg rats, with neuronal adenosine A_{2A} receptors overexpression had significantly increased risk developing lesions, when compared to Wt rats ($p<0.05$). This risk was increased not only for overall lesions, but specifically in the blood vessels, lung and adrenal gland ($p<0.05$).

Recent work published by Luisa Lopes laboratory at iMM-JLA, using the same transgenic rats (Tg (CaMKII-hA2AR)) showed that these animals have higher levels of corticosterone, decreased glucocorticoid receptors in the hippocampus and impaired function of the HPA axis, features of aging and Alzheimer's disease (Batalha et al., 2016). In normal conditions, activation of the HPA axis is controlled by parvocellular neurons located in the hypothalamus, which release corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal circulation, upon stimulation by stress or circadian drive. These factors cause the release of adrenocorticotrophic hormone (ACTH) into the systemic circulation and causes synthesis and secretion of glucocorticoids by the adrenal gland (Herman et al., 2012). Glucocorticoid secretion is negatively regulated by glucocorticoids at the level of the anterior pituitary gland. However, several regions of the brain, in particular the hippocampus, have abundant glucocorticoid receptors strongly involved in the negative feedback loop, therefore

having a crucial inhibitory role in the HPA axis (Mizoguchi et al., 2003). Under chronic stress conditions, hippocampal glucocorticoid receptors appear to be sensitive to high glucocorticoids, leading to a down-regulation of such receptors, therefore decreasing their inhibitory role in the HPA axis regulation and negative feedback loop (Mizoguchi et al., 2003). Thus, chronic exposure to glucocorticoids leads to cell death and hippocampal atrophy (Batalha et al., 2016). Therefore, adenosine A_{2A} overexpression leads to dysfunction of the HPA axis and triggers stress-like response in the hippocampus and high levels of circulating glucocorticoids.

Stress is defined as a state of threatened homeostasis provoked by psychological, environmental or physiological factors. When stress stimuli are prolonged and over exaggerated there is increased risk for both physical and psychiatric disorders. In fact, stress is a common risk factor of 75 to 90% of diseases, such as depression, cardiovascular diseases, inflammatory diseases, auto-immune disorders and wound healing (Liu et al., 2017). Although glucocorticoids reduce the expression of several proinflammatory cytokines, they also have a proinflammatory impact on the immune system (Liu et al., 2017). Glucocorticoids enhance the expression and function of "inflammasomes", which are cytoplasmic multi-protein complexes that sense exogenous and endogenous danger signals and cleave pro-inflammatory cytokines to mature cytokines such as IL-1β and IL-18 (Busillo, Azzams, & Cidlowski, 2011). Chronic stress is also associated with glucocorticoid resistance, whereas there is a decrease in the sensitivity of immune cells to glucocorticoids that usually terminate the inflammatory cascade (Miller et al., 2002). Without sufficient glucocorticoid regulation, the duration and intensity of the inflammatory response increases, therefore augmenting the risk of disease (Cohen et al., 2012). These facts could justify the correlation we saw, between A2AR OE in neurons and increased risk of disease in other organ systems. For instance, alveolar histiocytosis is characterized by the presence of macrophages in the alveoli and it was increased in the Tg rats. Chronic stress is known to induce glucocorticoid resistance in macrophages (Stark et al., 2011). One possibility is that chronic stress and high corticosterone levels could have enhanced the expression of the inflammasomes and glucocorticoid resistance, thereby increasing the duration and intensity of the activity of these macrophages.

The increased risk for development of vascular mineralization in transgenic rats was also observed ($p<0.05$). A link between vascular mineralization and poor cognitive function was observed by Bos and his team (Bos et al., 2012). According to their results, correlation was found between patients with poor cognitive function and vascular calcifications, although the underlying mechanism was not explained. Adenosine A_{2A} receptors are involved in neuroinflammation cognitive decline through activation of microglia cells (Chen et al., 2018). We could postulate that the increased incidence of vascular mineralization seen in rats with

compromised neuronal A2AR signaling may also be associated with poor cognitive function, in accordance with studies by Bos et al., but this needs further testing.

Chronic stress and higher levels of corticoids are linked to calcification of arteries and, although the mechanism is not yet fully understood, it is linked to HPA dysfunction (Evans et al., 2004; Evans & Ragolia, 2012; Hamer, Endrighi, Venuraju, Lahiri, & Steptoe, 2012). Evans and his team have postulated that ACTH produced during inflammatory states promotes osteochondrogenic mesenchymal cell differentiation contributing to the pathologic progression of calcified arteries (Evans & Ragolia, 2012). Glucocorticoids also promote chondrogenesis and osteoblastic differentiation pathway of mesenchymal cells, including vascular smooth muscle cells (Evans & Ragolia, 2012; Zhu, Rashdan, Chapman, Hadoke, & MacRae, 2016). Recent data show that vascular smooth muscle cells, the predominant cell type involved in vascular calcification, can undergo phenotypic transition to osteoblastic, chondrocytic and osteocytic cells in a calcified environment (Zhu et al., 2016). Thus, corticosterone acts in these cells and stimulates mineralocorticoid receptors therefore inducing pro-calcification effects (Zhu et al., 2016).

Tg rats have HPA dysfunction and higher levels of corticosterone, which can explain the higher risk for development of vascular mineralization observed. Furthermore, glucocorticoids exert complex actions on calcium mobilization and bone metabolism, regulating bone resorption and formation, intestinal calcium absorption and renal calcium excretion, which might also explain the reason why Tg rats have higher incidence and risk of developing lesions involving calcification pathways such as vascular mineralization, alveolar septa mineralization and intratubular mineralization.

The adrenal gland is also affected by chronic stress. Cortical cell vacuolation can be a secondary change to chronic stress, dysfunction of the HPA axis, and can be a result of excess of ACTH or corticosterone (Hoenerhoff et al., 2014). This could justify the fact that Tg rats have significantly higher risk of developing vacuolation of cortical cells.

Regarding neoplasia, mammary fibroadenoma and one single case of lymphoma were diagnosed within our sample. Neoplasia is very common in rats, and aged rats are often simultaneously afflicted with different types of tumors (Barthold, Griffey, & Percy, 2016). Hematopoietic neoplasms are rare in rats, when compared to mice. Within rats, these neoplasms have high incidence in aged Fisher rats. The incidence in Sprague- Dawley rats is relatively low when compared to other stains.

Mammary fibroadenoma was the most commonly diagnosed tumor in our population, with 5 cases in transgenic rats and 1 single case in a Wt rat. Mammary fibroadenomas are a relatively common finding in older female rats, particular Sprague Dawley rats and although rare, these

tumors can also occur in males (Barthold et al., 2016; Rudmann et al., 2012). The incidence of this specific neoplasms ranges from 7 to 40% in Sprague Dawley rats. There are several factors that play a role in the incidence of spontaneous mammary fibroadenomas such as age, sex, genetics, diet and endocrine factors (Barthold et al., 2016). For instance, restriction of food intake reduces the incidence of mammary fibroadenomas, and ovariectomized rats have also lower incidence of tumors. Prolactin levels can also affect the incidence of tumors since females with high prolactin levels have higher incidence of mammary tumors (Barthold et al., 2016; Rudmann et al., 2012) For this reason, some authors considered that pituitary adenomas that normally secrete high levels of prolactin, may be a major cause of mammary fibroadenomas, however unequivocal correlations have not been made (Barthold et al., 2016). In fact, the rats from our study did not show any macroscopic pituitary adenomas, in spite of the fact that levels of serum prolactin were not analyzed. Although no statistical significance was found between these tumors and transgenics, tendency for significance was associated to the fact that five of these tumors were found in transgenic rats and only one in a Wt rat. This could be linked to the chronic stress observed in the transgenic rats. Chronic stress has been linked with physiological and pathological disease outcomes, including several types of tumors. Stress-mediated immune modulation of cytokines including TNF-α, TGF-β, IL-1 and IL-6 have been suggested as indictors of cancer progression, metastasis and recurrence (Liu et al., 2017), which might explain the fact that the number of tumors in transgenic animals is higher than in Wild-type rats. However, more cases need to be analyzed in order to prove the link between transgenesis and mammary fibroadenomas.

5. Conclusion and future prospects

In conclusion, this study was focused on the spontaneous pathology arising a sample of ageing Sprague-Dawley rats, male and female, age ranging from 12 to 126 weeks, both Wt and Tg (CaMKII-hA2AR), with neuronal overexpression of human adenosine A_{2A} receptors. We diagnosed various age-associated lesions in several organs and tissues, and, most interestingly, were able to establish a statistical significant correlation between the onset of specific lesions and the genotype of the animals.

Neuronal over-expression of human A2AR in the rat seems to be associated with accelerated aging, as these animals have higher incidence and risk for developing ageing pathology in general, particularly vascular mineralization, inflammatory and degenerative lung lesions, and adrenal pathology. These could be both cause and consequence of the high chronic stress; disrupted HPA-axis, and increased values of circulatory corticosterone, already reported in these mutant rats.

In the future we hope to continue this work performing a more extensive phenotyping study, extending sample size and including also the analysis of central nervous system and characterizing the hematological, biochemical and endocrine profiles of these animals.

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